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**Nitrification in acid coniferous forests:
Some soils do, some soils don't**

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VRIJE UNIVERSITEIT

**Nitrification in acid coniferous forests:
Some soils do, some soils don't**

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad Doctor aan
de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus
prof.dr. L.M. Bouter,
in het openbaar te verdedigen
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Rully Adi Nugroho

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promotor: prof.dr. H.A. Verhoef
copromotoren: dr. W.F.M. Röling
 dr. A.M. Laverman

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To my parents in heaven

SUMMARY

Nitrification in acid coniferous forests: Some soils do, some soils don't

Nitrification is assumed to be rate-controlled by the activity of ammonia-oxidising bacteria (AOB). Therefore, AOB are considered to play a crucial role in the nitrogen cycle and nitrification in particular, and AOB were the main subject of the microbiological analysis described in this thesis. The general aim of this thesis was to elucidate the relation between the presence of AOB, environmental factors and nitrification rates in the soil layer of acid coniferous forests. Especially, the question why some acid forests showed nitrification while others did not was addressed in this thesis.

This thesis showed that nitrification occurred readily in some acid coniferous forest soils, but slowly in others. *Nitrosospira* cluster 2 was detected as the sole AOB in acid coniferous forest soils that showed high nitrification rates (Chapters 2 to 4). Conversely, AOB communities could not be detected in acid coniferous forest soils with low nitrification rates. The presence of *Nitrosospira* cluster 2 and differences in nitrification rates between nine Scots pine forest soils located throughout the Netherlands and Finland (Chapter 2) and in the Appelscha, the Netherlands forests containing pine, spruce, fir and larch tree species (Chapter 3) correlated with soil C/N ratio. Nitrification and *Nitrosospira* cluster 2 were exclusively detected in acid coniferous forest soils with C/N ratios below 26. Moreover, four tree-specific soils, i.e. pine, spruce, fir and larch forest soils were all sampled near Appelscha and located within a few kilometers from each other (Chapter 3). This indicates that differences in nitrification rates between nine Scots pine forest soils located throughout the Netherlands as well as Finland (Chapter 2) were also not related to geographic distances between these forests.

Atmospheric nitrogen deposition rather than geographical location appeared to be a more important affecting nitrification and the presence of AOB in the nine Scots pine forest soils (Chapter 2). AOB could not be detected and nitrification rates were nearly zero in soils receiving relatively low, according to Dutch standards, atmospheric nitrogen deposition. However, low atmospheric nitrogen deposition was not likely to be the sole factor determining nitrification (Chapter 3). The four different soil types, i.e. pine, spruce, fir and larch forest soils studied there came from around Appelscha, a location with low atmospheric nitrogen deposition. Still, three out of four forest soils revealed nitrification and the presence of AOB.

The low nitrification rates observed for some soils were not caused by the complete absence of AOB nor solely by C/N ratios but also by additional abiotic factors (unfavourable environmental conditions) (Chapter 4). Liming (to increase pH) and liming plus nitrogen amendment increased nitrification in soils that were known to have low nitrification rates. Conversely, nitrogen amendment did not affect nitrification rates in these soils. These results suggested that pH related factors, other than the effect of pH on ammonia availability (described below), had negative effects on the growth of AOB and their associative nitrifying activity in non-nitrifying soils. In soils treated with liming only, an effect of liming on C/N ratio was not observed, but still nitrification increased. On the other hand, when only ammonium was added, C/N ratio dropped but this did not induce nitrification. Presence and changes in the growth of AOB in soils

after treatment with liming and liming plus nitrogen amendment were apparent; not only nitrification rates increased but also AOB could be detected using molecular tools.

However, the low nitrification rates observed for some soils cannot be (solely) explained by unfavourable abiotic soil conditions. Chapter 5 showed that the onset of nitrification was observed after native or sterilised non-nitrifying soils were inoculated with fresh soil or soil slurry from the nitrifying soil. Conversely, when nitrifying soil was inoculated with non-nitrifying soil or soil slurry, nitrification was not inhibited. These indicate that abiotic factors or microorganisms that can potentially inhibit nitrification could not establish themselves in the nitrifying soils. These results falsified the hypothesis that interactions within the non-nitrifying soil microbial community prevent the onset of the growth and activity of AOB. Despite the occurrence of nitrification the bacterial community structure did not change to become nitrifying soil-like, suggesting that minor changes in community structure were sufficient to result in nitrification. General community profiling techniques targeting bacteria appear to be too insensitive to detect these minor microbial populations.

To summarise the effects of biotic and abiotic factors on the occurrence of nitrification in acid coniferous forest soils: presence of AOB, geographical location and ammonia availability are not particular factors that influence the occurrence of nitrification. Microbial interactions, C/N ratio, atmospheric N deposition, tree species and soil pH however do. The effects of microbial interactions, C/N ratio, atmospheric N deposition, tree species and soil pH are also related to other factors.

Very recently, the paradigm of AOB being primarily responsible for nitrification has become under siege. After the discovery of a unique ammonia monooxygenase gene on an archaeal-associated genome fragment in 2004, an ammonia-oxidising Archaea (AOA) has been isolated and it has been shown that AOA occur in high numbers in many habitats, including forest soils. In how far AOA are present and to which degree they contribute to nitrification in more acidic forest soils, such as investigated in this thesis, is unknown. To determine and understand the relative contribution of AOA and AOB to nitrification in acidic forest soils, biochemistry, physiology and ecology of AOA should be examined and compared to that of AOB.

SAMENVATTING

Nitrificatie in zure naaldbossen: Sommige bodems doen het, sommige bodems niet

Over het algemeen wordt aangenomen dat de snelheid van nitrificatie gereguleerd wordt door de activiteit van ammonia-oxyderende bacteriën (AOB). AOB worden dan ook gezien als een cruciale factor in de stikstofcyclus en in het bijzonder in nitrificatie. Om deze reden waren AOB het onderwerp van de microbiologische analyse die in dit proefschrift wordt beschreven. Het doel van dit onderzoek was het ophelderen van de relaties tussen de aanwezigheid van AOB, omgevingsvariabelen en de nitrificatiesnelheden in de bodem van zure naaldbossen. Vooral het feit dat sommige zure bosbodems nitrificatie laten zien en anderen niet, komt ter sprake in dit proefschrift.

In dit proefschrift wordt aangetoond dat in sommige zure bosbodems snelle nitrificatie plaatsvindt, terwijl in andere zure bosbodems het proces langzaam verloopt. In de bodems waar nitrificatie snel verloopt, werd alleen *Nitrosospira* cluster 2 aangetroffen (Hoofdstuk 2 tot en met 4). In de zure bosbodems waar een lage nitrificatiesnelheid werd gemeten, kon AOB niet worden aangetoond.

De aanwezigheid van *Nitrosospira* cluster 2 en verschillen in nitrificatie snelheden tussen negen Schotse grove dennenbossen in Nederland en Finland (Hoofdstuk 2) en in een bos nabij Appelscha, Nederland, dat Grove den, Taxus, Spar en Lariks bevat (Hoofdstuk 3) correleerde met de bodem C/N ratio. Nitrificatie en AOB werden alleen aangetroffen in zure bosbodems met een C/N ratio lager dan 26. De vier boom-specifieke bodems nabij Appelscha lagen binnen een paar kilometer van elkaar, maar lieten verschillen zien in nitrificatie en AOB aanwezigheid. Dit en de verschillen in nitrificatiesnelheden tussen de negen locaties verspreid over Nederland en Finland (Hoofdstuk 2) toont aan dat AOB en nitrificatie snelheden niet gerelateerd zijn aan geografische afstand.

Atmosferische depositie van stikstof heeft een groter effect op de nitrificatiesnelheid en op de aanwezigheid van AOB dan de geografische positie (Hoofdstuk 2). AOB konden niet worden waargenomen en nitrificatiesnelheden waren bijna nul in bodems die waren blootgesteld aan lage stikstof depositie uit de lucht (volgens de Nederlandse normen). Toch is de stikstofdepositie niet de enige bepalende factor (Hoofdstuk 3). De vier verschillende bodemtypes (Grove den, Taxus, Spar en Lariks) afkomstig uit de omgeving van Appelscha, liggen allemaal in een regio met een lage stikstofdepositie. Toch werd bij drie van de vier bodems nitrificatie en AOB aangetoond.

De lage nitrificatiesnelheden die zijn gemeten in sommige bodems werden niet veroorzaakt door de totale afwezigheid van AOB noch alleen door C/N ratio's maar ook door aanvullende abiotische factoren (ongeschikte omgevingscondities) (Hoofdstuk 4). Bekalken (verhogen van de zuurgraad) en bekalken in combinatie met een extra stikstof gift verhoogden de nitrificatiesnelheid in bodems waarvan is aangetoond dat de nitrificatiesnelheid normaalgesproken laag is. Het toevoegen van alleen extra stikstof had geen effect op de nitrificatiesnelheid. Deze resultaten geven aan dat pH gerelateerde factoren, anders dan het effect van pH op ammonium beschikbaarheid (hieronder beschreven), negatieve effecten hadden op de groei van AOB en hun nitrificatie activiteit in niet-nitrificerende bodems. In bodems waar alleen bekalkt is, werd geen effect op de C/N ratio waargenomen en toch nam de nitrificatie snelheid toe. Nitrificatie

werd niet geïnduceerd wanneer de C/N ratio naar beneden wordt bijgesteld wordt door toevoeging van ammonium. De effecten van bekalken en bekalken in combinatie met stikstoftoediening op de nitrificatiesnelheid en de groeisnelheid van AOB waren overduidelijk, niet alleen de nitrificatiesnelheid nam toe maar ook AOB konden worden aangetoond met behulp van moleculaire technieken.

Toch kan de lage nitrificatiesnelheid die in sommige bodems werd aangetoond niet (geheel) worden verklaard aan de hand van ongeschikte abiotische bodemcondities. In hoofdstuk 5 is te lezen dat nitrificatie startte in gesteriliseerde, niet-nitrificerende bodems nadat ze waren geïnoculeerd met verse nitrificerende bodem of bodem-slurry daarvan. Omgekeerd, wanneer nitrificerende bodems werden geïnoculeerd met niet-nitrificerende bodem of een slurry daarvan, vond geen inhibitie plaats. Deze bevindingen geven aan dat abiotische factoren of micro-organismen die in potentie nitrificatie kunnen remmen, zichzelf niet kunnen vestigen in nitrificerende bodems.

Deze resultaten falsifiëren de hypothese waarin wordt gesteld dat niet-nitrificerende bodems een microbiële gemeenschap bevatten die de groei en de activiteit van AOB kunnen voorkomen. De samenstelling van de bacteriële gemeenschap veranderde niet in de richting van een gemeenschap afkomstig uit een nitrificerende bodem. Dit geeft aan dat kleine wijzigingen in de soortensamenstelling voldoende kunnen zijn om nitrificatie op te starten. De algemene technieken om de samenstelling van de bodemgemeenschap in beeld te brengen, blijken niet gevoelig genoeg te zijn om deze minieme verschuivingen te detecteren.

Samengevat zijn de effecten van biotische en abiotische factoren op het voorkomen van nitrificatie in zure bodembodems de volgende: aanwezigheid van AOB, geografische ligging en ammoniabeikbaarheid zijn geen factoren met een grote invloed op het voorkomen van nitrificatie. De boomsoort is van groot belang op de nitrificatie. Microbiële interacties, C/N ratio, atmosferische stikstofdepositie, boomsoort en de zuurgraad van de bodem zijn ook van belang maar zijn ook gerelateerd aan andere factoren.

Het paradigma dat stelt dat AOB de primaire factor zijn in nitrificatie is zeer recentelijk ter discussie komen te staan. In 2004 werd een uniek ammonium-monooxygenase gen ontdekt op een fragment van het genoom van een Archaea. Snel daarna werden ammonia-oxyderende Archaea (AOA) geïsoleerd. Deze organismen komen in hoge dichtheden voor in veel habitats, waaronder bosbodems. In hoeverre AOA aanwezig zijn en bijdragen aan nitrificatie in de zure bodems van naaldbossen zoals onderzocht in dit proefschrift, is onbekend. Om vast te stellen wat de relatieve bijdrage van AOA en AOB aan de nitrificatie in zure bosbodems is, moet de biochemie, de fysiologie en de ecologie van AOA worden onderzocht en vergeleken met die van AOB.

CHAPTER 1

GENERAL INTRODUCTION

Introduction

Nitrification is a key process in the global nitrogen cycle, resulting in nitrogen loss from terrestrial ecosystems, eutrophication of surface water and groundwater, and the production of atmospherically active trace gases (greenhouse gases). Scientific interest in nitrification, the oxidative process which converts ammonia to nitrite and subsequently to nitrate, in acid forest soils (pH < 6.0) has increased in the last years. Nitrification in acid forest soils is important because it affects the distributions of the different forms of inorganic nitrogen in the soil and therewith nitrogen availability; the nitrate that is formed is both more readily available to plants and more easily leached from soil than ammonium (Vitousek et al., 1982). In addition, nitrification is a soil-acidifying process (van Miegroet and Cole, 1985).

Studies have shown that nitrification can occur in acid coniferous forest soils (Bäckman and Klemetsson, 2003; Bottomley et al., 2004; Degrange et al., 1998; Hart, 2006; Killham, 1990; Laverman et al., 2002; 2005; Stark and Hart, 1997). Even though, for many years the process of nitrification was thought to play a minimal role in nitrogen cycling in coniferous forest ecosystems (Mintie et al., 2003), because several coniferous forest soil factors were considered suboptimal to nitrifying microorganisms and their activity. These factors comprise soil acidity, high C/N ratios, low nitrogen availability, and/or presence of allelochemical compounds (de Boer and Kowalchuk, 2001; Kowalchuk and Stephen, 2001).

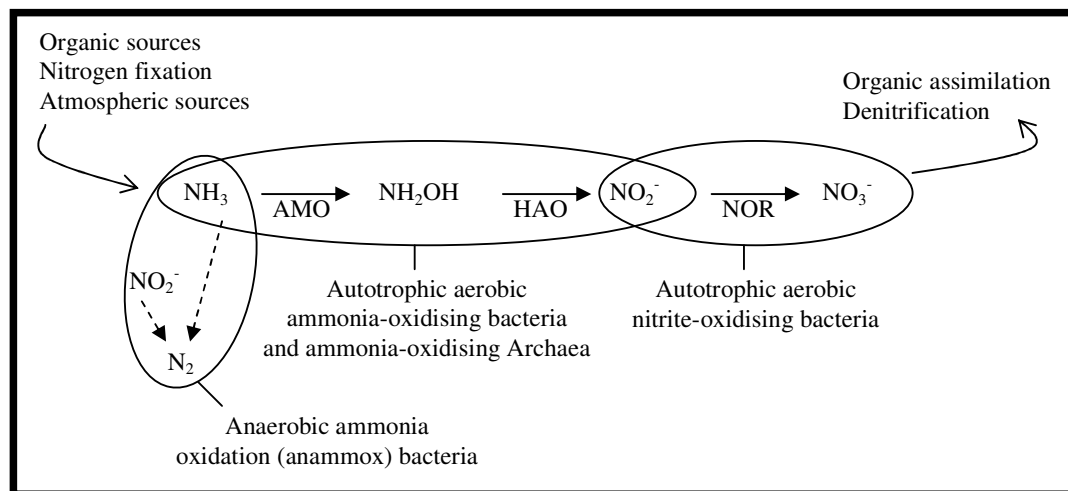


Figure 1. Autotrophic ammonia oxidation during nitrification. Ammonia-oxidising organisms convert ammonia to nitrite through hydroxylamine using the enzymes ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO). Autotrophic nitrite-oxidising bacteria subsequently use the enzyme nitrite oxidoreductase (NOR) to convert nitrite to nitrate, which can be assimilated or subjected to denitrification processes. In anaerobic environments, ammonia can be converted to molecular nitrogen by the anammox process by several enzymatic steps, represented by dashed arrows (Nicol and Schleper, 2006).

In most ecosystems, including acid coniferous forest soils, nitrification was long considered to be carried out mainly by aerobic autotrophic bacteria (de Boer et al., 1995; Persson and Wirén, 1995; Prosser, 1989). Aerobic autotrophic nitrification is a two-step process, the first being oxidation of ammonia to nitrite, followed by the

oxidation of nitrite to nitrate. The two steps are performed by different groups of aerobic bacteria, the ammonia-oxidising bacteria (AOB) and nitrite-oxidising bacteria (Prosser, 1989), respectively (Fig. 1). Autotrophic bacteria that can oxidise ammonia directly to nitrate have not been described, although recently Costa et al. (2006) postulated that bacteria that completely oxidise ammonia to nitrate, referred to as complete ammonium oxidation (comammox), exist in the environment. When the experimental work described in this thesis started, nitrification was assumed to be rate-controlled by the activities of AOB. Therefore, AOB were considered to play a crucial role in the nitrogen cycle and nitrification in particular, and they are therefore the main subject of the microbiological analysis described in this thesis.

Distinct from autotrophic nitrification, ammonia can also be aerobically oxidised by a number of heterotrophic fungi and bacteria (Killham, 1990; Prosser, 1989). It is uncertain whether heterotrophic microorganisms nitrify via an inorganic pathway ($\text{NH}_4^+ \rightarrow \text{NH}_2\text{OH} \rightarrow \text{NOH} \rightarrow \text{NO}_2^- \rightarrow \text{NO}_3^-$), perhaps involving hydroxylamine and nitrite as likely intermediates, or via an organic pathway ($\text{RNH}_2 \rightarrow \text{RNHOH} \rightarrow \text{RNO} \rightarrow \text{RNO}_2 \rightarrow \text{NO}_3^-$), possibly involving oxidation of an amine or amide to a substituted hydroxylamine and with subsequent oxidation to a nitroso- and then to a nitro-compound, or via a combined inorganic/organic pathway (Killham, 1990). In some acidic coniferous soils, heterotrophic nitrifiers have been considered to be responsible for nitrification (Brierley et al., 2001; Duggin 1991; Jordan et al., 2005; Killham, 1987; 1990; Lang and Jagnow, 1986; Papen and von Berg, 1998; Stroo et al., 1986). Heterotrophic nitrifiers might be of significance in acidic forest soils, where their large numbers or high biomass might compensate for their relative inefficiency while autotrophic nitrification might be hindered by the low pH (Kuenen and Robertson, 1988). However, results from a large number of studies, applying specific inhibitors of autotrophic nitrification, have revealed that heterotrophic nitrification does not play an important role in acidic coniferous forest soils (Burns and Murdoch, 2005; De Boer et al., 1992; Laverman et al., 2000; Martikainen et al., 1993; Paavolainen and Smolander, 1998; Pedersen et al., 1999; Pennington and Ellis, 1993; Rudebeck and Persson, 1998; Ross et al., 2004; Stams et al., 1990; Stark and Hart, 1997).

Alternatively, ammonia can be oxidised by anaerobic ammonia oxidation (anammox) bacteria (Jetten et al., 1997; Strous et al., 2002). Bacteria responsible for anammox were discovered in the early 1990s in a denitrifying pilot plant in Delft, The Netherlands (Strous et al., 1999; Strous and Jetten, 2004). Under completely anoxic conditions NH_4^+ is oxidised with NO_2^- as electron acceptor to N_2 and small amounts of NO_3^- (Jetten et al., 1999). Anammox is known to be active at pHs between 6.7 and 8.3, with an optimum at pH 8.0 (Jetten et al., 1999). Anammox bacteria grow extremely slow and are difficult to culture; in fact, pure cultures have not yet been obtained from the enrichments (Strous et al., 1999; Strous and Jetten, 2004). All known anammox bacteria form a monophyletic branch of the *Planctomycetales* and are, therefore, phylogenetically completely unrelated to the aerobic AOB (Schmid et al., 2000; Schmid et al., 2003; Strous et al., 1999; Strous and Jetten, 2004). Currently, three known genera of anammox bacteria: *Brocadia*, *Kuenenia* and *Scalindua* have been found in wastewater treatment systems and marine ecosystems (Kuypers et al., 2003; Mulder et al., 1995; Risgaard-Petersen et al., 2004; Third et al., 2005; Trimmer et al., 2003). Data on nitrification in acid forest soils by anammox bacteria are currently not available. The

aerobic status of soils, the low soil pHs and the low *in situ* concentrations of nitrite suggest a minor role of anammox in ammonia-oxidation at most.

Very recently, the paradigm of AOB being primarily responsible for nitrification has become under siege. High numbers of active ammonia-oxidising Archaea (AOA) were discovered in calcareous grassland soil (Treusch et al., 2005) and most recently in agriculture and grassland soils with pH 5.5 to 7.3 (Leininger et al., 2006). These organisms, affiliated to the phylum Crenarchaeota, have ammonia monooxygenase (AMO) genes, the key enzyme of AOB, which are only distantly related to those from Proteobacteria (Treusch et al., 2005). Further insight into potential metabolic capabilities of Archaea comes from recent work on microbial genomic diversity in the Sargasso Sea by shotgun DNA sequencing (Venter et al., 2004). Based on their discovery of an ammonia monooxygenase gene on an archaeal-associated scaffold, it was suggested that some Archaea may be capable performing chemoautotrophic nitrification (Venter et al., 2004). The first ammonia-oxidising archaeon, *Nitrosopumilus maritimus*, was isolated from a marine aquarium (Könneke et al., 2005) and shown to be chemolithoautotrophic and able to oxidise ammonia to nitrite (Könneke et al., 2005). It also seems that it grows at similar rates and density as cultured AOB (Könneke et al., 2005). Although AOA outnumber AOB in most investigated soils so far, it should be noted that these soils had a pH of at least 5.5 (Leininger et al., 2006) and that currently it has not been proven that these higher AOA numbers also translate to a high contribution of AOA to nitrification rates.

Measuring nitrification rates in coniferous forest soils

Accurate measurements of nitrification rates in forest soil, including acid coniferous forest soils, are needed to provide insight into soil fertility and ecosystem function, and are necessary to be able to assess potential effects of ecosystem management, climate change and other perturbations. Nitrification assays are usually conducted under conditions where plant uptake, leaching, microbial assimilation (conversion of nitrate to cellular organic nitrogen via ammonia), microbial dissimilation (oxidation of carbon compounds at the expense of nitrate which acts as an alternative electron acceptor to oxygen), and denitrification are suppressed (Robertson et al., 1999). Under such conditions nitrification can be estimated from accumulation of nitrate or the loss of ammonium.

Nitrification rates have been measured using many different sampling techniques, incubation conditions and incubation times (see Binkley and Hart (1989) for a comprehensive review on the methodology). The variety of techniques available for measuring nitrification rates can be classified into four main groupings: (1) *in situ* incubations of enclosed soils, in which inorganic nitrogen accumulation is measured at the end of a 2-6 weeks, or months incubation period (e.g. de Boer et al., 1993; Fenn et al., 2005; Hart, 2006; Jussy et al., 2004; Tietema et al., 1993; Vestgarden et al., 2003); (2) laboratory incubations under standard moisture and temperature conditions in which inorganic nitrogen accumulation is monitored at 7-30 days intervals, for up to a year or more (e.g. Carnol et al., 2002; Kanerva et al., 2006; Laverman et al., 2005); (3) laboratory- or field-based isotopic incubations during which changes in a ^{15}N -labeled

inorganic nitrogen pool are measured over the course of a 1-3 day incubation (e.g. Perakis et al., 2005; Ross et al., 2004; Scowcroft et al., 2004); and (4) laboratory nitrification rates measured over a short period (~1 day) (e.g. Bäckman and Klemetsson, 2003; Ross et al., 2006).

There are many possible permutations of procedures within these four major groups. These include the degree of soil disturbance (intact cores versus sieved soils), incubation temperatures (*in situ* temperature fluctuations versus a constant temperature such as 25 °C), incubation moisture (field moisture at the time of sampling versus some proportion of water-filled pore space or field capacity), substrate enrichment (absence versus presence of added ammonium), and – for laboratory incubations – aeration status (ranging from added sand to increase aeration to anaerobic slurries to eliminate oxygen altogether).

Overall, no method provides a clear, accurate assessment of the nitrification rates in a forest soil (Binkley and Hart, 1989). The selection of the appropriate method for each study needs to be based on the objectives of the study, especially on the component of the nitrogen cycle that are expected to be of interest. One of the most frequently used methods of nitrification assessment is aerobic incubation under controlled environmental conditions (Binkley and Hart, 1989). Although the standardised conditions are artificial, they provide an opportunity for comparisons across sites and across studies that would not be possible under varying conditions (Binkley and Hart, 1989).

Detecting ammonia-oxidising bacteria (AOB) in soils

As stated above, during the experimental stage of the research described in this thesis, nitrification was assumed to be rate-controlled by the activities of ammonia-oxidising bacteria (AOB), therefore, methods to analyse AOB community were critical. Conventional approaches to analyse AOB communities employ cultivation-based methods. It is now generally accepted that cultivation-based techniques provide an incomplete picture of natural AOB communities, resulting from the selective nature of laboratory media and incubation conditions, competition for nutrients by other organisms during incubation and difficulties in creating media and growth conditions that faithfully reproduce those found in natural environments (Prosser and Embley, 2002; Smith et al., 2001). The difficulties in the isolation of pure strains of AOB and the limited number of morphological characteristics for identification and classification also hampers an understanding of their important role in carrying out ammonia oxidation (Prosser and Embley, 2002; Smith et al., 2001). The major difficulties with respect to isolation are exacerbated slow growth of AOB in liquid culture and on solid media, difficulties in separating AOB from heterotrophic contaminants and the dying out of AOB with repeated subculturing (Prosser and Embley, 2002). Using morphological criteria, AOB are classified into five different genera *Nitrosomonas*, *Nitrosococcus*, *Nitrospira*, *Nitrosolobus* and *Nitrosovibrio* (Koops et al., 2003).

In recent years, the application of molecular approaches has become popular as these do not require enrichment and isolation of pure cultures, thereby removing the major problems discussed above. Analysis of 16S rRNA gene sequences was first applied to a

limited number of AOB by Woese et al. (1984; 1985), with subsequent analysis of sequences from eleven pure cultures by Head et al. (1993). Molecular analysis of AOB communities comprises analysis of amplified 16S rRNA genes by cloning, followed by sequencing and fingerprinting by methods such as denaturing and temporal temperature gradient electrophoresis (DGGE, TTGE; Muyzer et al., 1993; Muyzer and Smalla, 1998). These analyses have led to the recognition of seven 16S rRNA beta-subclass AOB sequence clusters (Stephen et al., 1996). Koops et al. (2003) have expanded the subdivision of AOB into nineteen sequence clusters by including environmentally retrieved 16S rRNA gene sequences.

Analysis of the 16S-23S rRNA intergenic spacer region (ISR) sequences in combination with 16S rRNA gene sequences has been applied as an alternative technique to clarify the phylogeny of AOB (Aakra et al., 1999; 2001; Gürtler and Stanisich, 1996). Bacterial rRNA genes are commonly organized in operons which contain different genes separated by spacers (Gürtler and Stanisich, 1996). Normally, the gene organization of bacterial rRNA gene clusters is in the order 5'-16S rRNA-ISR-23S rRNA-5S rRNA-3'. The length of the spacers, in which tRNA-encoding genes can frequently be found, varies considerably between species (200-1500 bp) (Gürtler and Stanisich, 1996). Similarity of ISR sequences of the AOB is low (42.9-96.2%) compared with 16S rRNA sequence similarity (occasionally more than 90% within the genus *Nitrosospira*), and therefore the ISR sequences are valuable as a complementary phylogenetic tool in combination with 16S rRNA gene sequences (Aakra et al., 1999).

The gene encoding subunit A of ammonia monooxygenase (*amoA*) is nowadays often applied as an alternative phylogenetic marker of AOB (Rotthauwe et al., 1997; Purkhold et al., 2000; Ivanova et al., 2000). Nine *amoA* classes are distinguished among the beta subclass of AOB (Avrahami and Conrad, 2003). The *amoA* gene is present in all autotrophic AOB and is believed to contain sufficient information to make phylogenetic inferences based on its sequence (Purkhold et al., 2000; Rotthauwe et al., 1997). The *amoA* gene has two advantages over 16S rRNA genes for comparisons of genetic diversity (O'Mullan and Ward, 2005). First, *amoA* encodes a protein involved directly in ammonia oxidation; therefore genetic differences are more likely to be of functional importance to the nitrification process. Secondly, the rate of molecular divergence in *amoA* is expected to exceed that of 16S rRNA genes, allowing greater resolution of genetic differences in natural populations. The *amoA* gene maybe subject to more intense selection than rRNA because it determines an essential function; this could diversify the gene by selection under different conditions, but also stabilize the gene sequence for those segments essential for the function. Generally, 16S rRNA and *amoA*-based trees possess congruent topologies (Koops et al., 2003). Other genes encoding subunits of ammonia monooxygenase protein are termed *amoB* and *amoC* (Calvó and Garcia-Gil, 2004; Klotz et al., 1997; Norton et al., 2002). These have been proposed by these researchers as an alternative target for molecular analysis of AOB, as well as for providing new insight into the classification of this group.

Alternative techniques to 16S rRNA and *amoA* genes sequencing have also been suggested to clarify the phylogeny of many β -proteobacterial AOB, such as the gene encoding hydroxylamine oxidoreductase (HAO) (Bergmann et al., 2005), the second enzyme in the nitrification pathway which oxidises hydroxylamine to nitrite (Whittaker

et al., 2000). Ribulose 1,5-biphosphate carboxylase/oxygenase (RubisCO) gene (Utåker et al., 2002) is another alternative. The RubisCO enzyme is the key enzymes of the Calvin-Benson-Bassham (CBB) cycle for assimilating CO₂ (Utåker et al., 2002).

Gene sequences of glyceraldehyde 3-phosphate dehydrogenase (*gap*) and phosphoglycerate kinase (*pgk*) are other proposed phylogenetic markers for the genera *Nitrosolobus*, *Nitrosospira* and *Nitrosovibrio* (Ida et al., 2005). These two enzymes have functions in both the glycolytic pathway and the reductive pentose phosphate cycle. However, these *gap* and *pgk* genes are not unique to AOB since they appear in many other organisms. In addition, the topology of the *gap* tree was different from that generated by 16S rRNA results (Ida et al., 2005). Likewise, to a lesser extent for RubisCO, many other autotrophic organisms possess this enzyme (Tolli and King, 2005; Utåker et al., 2002). Furthermore, information on all these alternatives genes is still scarce to clarify the phylogeny of AOB.

The application of molecular tools as discussed above has significant advantages for the analysis of AOB communities. Apart from that analysis can be carried out without enrichment and isolation of pure cultures, acquisition of sequence data is more rapid than conventional isolation procedures. On the other hand, the mere demonstration of sequences does not prove that the organism carrying these sequences actually carries out the functions implied. Therefore, genetic screening must always be complemented by functional studies to demonstrate a role in nitrogen cycle.

Physiological characteristics of ammonia-oxidising bacteria (AOB) and their ecological significance

At present, molecular techniques cannot (yet?) provide detailed insight into the physiological characteristics of ammonia-oxidising bacteria (AOB). For this reason, culturing and isolation are still important. It is, therefore, a challenge for the future to link molecular and traditional approaches to establish the relationship between AOB community structure, nitrification and associated ecosystem processes and to determine the significance of the diversity observed in natural communities.

Compared to other functional groups in soil, AOB have a low diversity, slow growth rate and are sensitive to acidity and to a whole range of chemicals, such as tannins, polyphenolics, monoterpenes, hydrocarbons, and heavy metals (de Boer and Kowalchuk, 2001; Kowalchuk and Stephen, 2001; Prosser, 1989). Incubation on solid medium for several weeks is required for the production of microscopic colonies (Prosser and Embley, 2002). The growth of pure cultures of AOB in liquid batch culture does not occur at pH <6.5 because of increased ionisation of ammonia (Allison and Prosser, 1991). Despite this, autotrophic nitrification has been reported in acid soils at pH 3.5 (de Boer et al., 1988; de Boer and Kowalchuk, 2001). A number of explanations for autotrophic nitrification in acid soils have been proposed, including growth and/or activity on the surface of particulate material (Allison and Prosser, 1993), or in aggregates (de Boer et al., 1991), and ureolytic activity (de Boer and Laanbroek, 1989; Allison and Prosser, 1991). Another explanation is the existence of strains adapted to low pH environments (de Boer et al., 1995).

Although the basic metabolism is more or less uniform within physiologically defined groups of autotrophic AOB, physiological differences exist between the distinct representatives (Koops and Pommerening-Röser, 2001). All species of autotrophic AOB use ammonia as the sole energy source (Koops and Pommerening-Röser, 2001). However, the substrate affinity (K_s value of the ammonia-oxidising system) differs significantly between species, which affects competition between species. Strains of *Nitrosomonas ureae* and *Nitrosomonas oligotropha* possess very high affinity for ammonia (K_s of 1.9 and 4.2 μM) (Stehr et al., 1995), and, recently, it was shown that a strain of *Nitrosomonas oligotropha* outcompeted a strain of *Nitrosomonas europaea* (K_s of 30-60 μM) when both were grown in an ammonium-limited chemostat (Bollmann et al., 2002). In the case of *Nitrospira* species, affinity constants for ammonia of isolated strains ranged between 6 and 11 μM (Jiang and Bakken, 1999). The determined affinity constant for ammonia obtained for *Nitrospira* sp. AV (cluster 3) was in the range of 0.8 to 1.4 μM (Taylor and Bottomley, 2006), which was more similar to the range of values found for isolates of *Nitrosomonas ureae* and *Nitrosomonas oligotropha* (1.9 to 4.2 μM NH_3) (Bollmann and Laanbroek, 2001; Koops and Pommerening-Röser, 2001).

Urea as ammonia source for ammonia-oxidising bacteria (AOB)

Several researchers have investigated nitrification in acid soils at pHs around 4, where free ammonia concentrations are very low because of its ionisation to ammonium. Urea from animal wastes and urea fertilisers might be the only energy source for AOB (de Boer et al., 1988; de Boer et al., 1989; de Boer et al., 1992; de Boer and Laanbroek, 1989; Pennington and Ellis, 1993; Troelstra et al., 1992; Walker and Wickramasinghe, 1979). One of the prevailing major mechanisms explaining autotrophic nitrification in acid soils is the ability of many AOB to hydrolyze urea. Urea can be taken up by cells at a low pH through diffusion and then hydrolysed intracellularly to ammonia (Burton and Prosser, 2001). A proportion of this ammonia diffuses into the medium, but some remains in the cell and oxidises to nitrite, which then also diffuses into the medium (Burton and Prosser, 2001). Urea has been found to stimulate autotrophic nitrification in Dutch forest soil (de Boer et al., 1992).

Many AOB species, but not all, are able to use urea as an ammonia source (Burton and Prosser, 2001; Koops and Pommerening-Röser, 2001; Koops et al., 1991). Within the group of AOB located in the β -subdivision *Proteobacteria*, there is an interesting difference between the two main clusters, the *Nitrospira* and *Nitrosomonas* clusters. For all species of the *Nitrospira* cluster, both urease-positive and urease-negative strains have been observed (Koops and Pommerening-Röser, 2001). Within the *Nitrosomonas* cluster, urease activity has not been observed for all species and, in contrast to the *Nitrospira* cluster, nearly all isolates of a given urease-positive species revealed this property in laboratory experiments (Koops and Pommerening-Röser, 2001).

Outline of this thesis

The principal aim of this thesis was to elucidate the relation between the presence of ammonia-oxidising bacteria (AOB), environmental factors, and nitrification rates in the fragmentation layer of acid ($\text{pH} < 3.0$) coniferous forest soils. Especially the question why some acid forests show nitrification while others do not was addressed in this thesis.

The work described in Chapter 2 was aimed to test the hypothesis that soils with different NH_4^+ -N concentrations contain different autotrophic AOB and that this difference correlates to N transformation rates. In Chapter 3, the hypothesis was that the presence of AOB and their nitrifying activity were affected by tree species. In these two studies (Chapter 2 and 3), environmental factors, such as C/N ratio of the fragmentation layer and atmospheric N deposition, as well as tree species, through their effects on soil C/N ratios, were found to affect the presence of the AOB, which may explain the big differences in nitrification rates between the different soils. Sequencing confirmed that exclusively *Nitrosospora* cluster 2-like 16S rRNA and *amoA* genes sequences were detected in soils with high nitrification rates. However, in soils with low nitrification rates AOB-like sequences could not be detected.

Because of the complexity in the patterns of occurrence of nitrification and the process-controls on nitrification in coniferous forest soils, some of the main physicochemical and biological factors which dominate control of nitrification were examined to improve the understanding of the process in these soils. Whether low nitrification rate has a biotic cause (complete absence of AOB) or an abiotic cause (unfavourable environmental conditions) was investigated in Chapter 4. Two soils strongly differing in net nitrification were compared: one soil with a low nitrification rate and another soil with a high nitrification rate were subjected to liming and/or ammonium amendment treatments. The results revealed that liming, rather than ammonium amendment, stimulated the growth of AOB and their nitrifying activity in soils known to have low nitrification rates. This study suggests that low nitrification rates in acidic Scots pine forest soils are due to pH-related factors.

Finally, the hypothesis that general microbial community structure and interactions between members of the microbial community in acid Scots pine forest soils with low nitrification rate prohibited the growth of autotrophic ammonia-oxidising bacteria (AOB) and their nitrifying activity was examined in Chapter 5. Here, native and sterilised soils known to have low nitrification rates were augmented with fresh soils or soil slurries from nitrifying soil, and vice versa. This study showed that augmentation with nitrifying soils induced nitrification in previously non-nitrifying soils, although no significant changes in bacterial community structure were observed. In sterilised soils, the inoculum determined the occurrence of nitrification and bacterial community structure. This study demonstrated that low nitrification rates in acid Scots pine forest soils cannot be (solely) explained by unfavourable abiotic soil conditions. The occurrence of nitrification after augmentation of native soils known to have low nitrification rates with nitrifying soil or soil slurry falsified the hypothesis that interactions within the non-nitrifying soil microbial community prevent the onset of the growth and activity of AOB.

Chapter 6 provides a general discussion on the experimental chapters (chapters 2 to 5) of this thesis. Results are also discussed in relation to the recent discovery of the ammonia-oxidising Archaea (AOA) and to the potential important role of AOA in the nitrogen cycle in acid coniferous forest soils.

CHAPTER 2

PRESENCE OF *NITROSOSPIRA* CLUSTER 2 BACTERIA CORRESPONDS TO N TRANSFORMATION RATES IN NINE ACID SCOTS PINE FOREST SOILS

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Abstract

The relation between environmental factors and the presence of ammonia-oxidising bacteria (AOB), and its consequences for the N transformation rates were investigated in nine Scots pine (*Pinus sylvestris* L.) forest soils. In general, the diversity in AOB appears to be strikingly low compared to other ecosystems. *Nitrosospira* cluster 2, as determined by temporal temperature gradient electrophoresis and sequencing, was the only sequence cluster detected in the five soils with high nitrification rates. In the four soils with low nitrification rates, AOB-like sequences could not be detected. Differences in nitrification rates between the forest soils correlated to soil C/N ratio (or total N) and atmospheric N deposition

Keywords: nitrification, ammonia-oxidising bacteria, acid Scots pine forests, environmental factors

Introduction

Nitrification is carried out in most ecosystems by autotrophic bacteria and this process is often considered rate limited by the activities of ammonia-oxidising bacteria (AOB). These bacteria are responsible for the oxidation of ammonia to nitrite by the enzymes ammonia monooxygenase and hydroxylamine oxidoreductase (de Boer and Kowalchuk, 2001; Kowalchuk and Stephen, 2001).

All known terrestrial AOB to date belong to a monophyletic assemblage of *Nitrosospira* and *Nitrosomonas* in the β -subdivision *Proteobacteria* (Kowalchuk and Stephen, 2001; Stephen et al., 1996). This assemblage originally consists of seven 16S rRNA gene sequence clusters (Stephen et al., 1996). Koops et al. (2003) have expanded the subdivision of AOB by including environmentally retrieved 16S rRNA gene sequences. Molecular analysis revealed that *Nitrosospira* cluster 2 dominated in acid soils (Kowalchuk et al., 2000b; Laverman et al., 2001; Stephen et al., 1996). *Nitrosospira* cluster 3 and *Nitrosomonas* cluster 7 were major AOB in tilled and fertilised soils (Bruns et al., 1999; Phillips et al., 2000; Webster et al., 2002), while *Nitrosospira* cluster 0 dominated under undisturbed and unfertilised grassland (Kowalchuk et al., 2000a; Webster et al., 2002). Generalisation regarding the relationship between clusters and environmental variables cannot be made as other 16S rRNA gene (rDNA)-based studies showed that *Nitrosomonas europaea* cluster 7 can also be present in acid soils (Carnol et al., 2002) and in unfertilised grasslands (Webster et al., 2002), and *Nitrosospira* cluster 3 in both fertilised and unfertilised grassland (Webster et al., 2002). Therefore, it remains unclear how the composition of AOB communities varies due to different environmental factors.

Our aim is to improve the understanding on the relationship between the presence of AOB and environmental factors in acid Scots pine forest soils, and the consequences for the *in situ* N transformation process. We therefore (i) quantified the N transformation rates, i.e. net mineralisation, net nitrification and net ammonification rates in nine Scots pine forest soils with different NH_4^+ -N concentrations; (ii) established correlations between soil properties, i.e. total C, total N, C/N ratio, Ca content, pH, initial NO_3^- -N and NH_4^+ -N concentrations and N transformation rates and (iii) tested the hypothesis that soils with different NH_4^+ -N concentrations contain different autotrophic AOB and that this difference correlates to N transformation rates.

A culture-independent method was used to examine the presence of AOB in nine acid Scots pine forest soils that have been subjected to different levels of N deposition for many years. Molecular biological approaches have significant advantages for analysis of *in situ* AOB communities (Kowalchuk et al., 1997), as analysis can be carried out without enrichment and isolation of pure cultures.

Materials and Methods

Field site and forest floor collection. Eight sites located in the Netherlands and one site in Finland (Table 1) were chosen to represent a large range in NH_4^+ -N concentrations. The forest floor (fragmentation (F) layer) of Scots pine (*Pinus sylvestris* L.) stand utilized in this study was collected in June 2002. At each site, ten samples

(15×20 cm) of the F layer were collected randomly from an area of approximately 5×5 m, then pooled in a clean plastic bag and returned to the laboratory. Pooling was necessary to make the soil more homogeneous. The field-moist soils were immediately passed through a 4 mm sieve in the laboratory and homogenized by hand. Sieving through 2 mm sieves has hardly any effect on mineralisation (Kristensen et al., 2003). Sub-samples of 30 g of the sieved material were stored at -20°C until molecular analysis. The remaining sieved material was kept at 5°C, to minimise changes in initial conditions across the soils, for less than a week until further analysis on soil characteristics and N transformation rates. In this study the F layer was chosen, as in this layer, compared to the litter (L) layer and mineral soil, less spatial variation was observed in mineralisation and nitrification rates and temporal variation in these rates was not significant (Laverman et al., 2000). In addition to that, denitrifying capacity was lowest in the F layer (Laverman et al., 2001).

Soil characteristics analysis. Sub-samples of the sieved material were analysed for total N and total C on a Carlo Erba Strumentazione elemental analyser (model 1106), and Ca content on an atomic absorption spectrophotometer (AAS) (Perkin Elmer, model 1100B). Moisture contents of the soils were determined by drying fresh samples at 50°C for 3 days. Other sub-samples were extracted with 1 M KCl (15 g field-moist soil:100 ml of 1 M KCl) on a shaker at 200 × g for 1.5 h and filtered through filter paper (Schleicher & Schuell, 595.5). Extractable NH_4^+ -N and NO_3^- -N concentrations were measured on an autoanalyser (Skalar SA-40), and pH was measured using a Consort P907 pH meter.

N transformation rate determination. N transformation rates were determined using the method described by Laverman et al. (2000). Before the N transformations experiment, sub-samples for incubation were brought to 65% moisture content by adding demineralised water. Two 250 ml screw-cap bottles were filled with 15 g fresh material each. One bottle was capped with a regular cap and the other with a septum-contained cap. Acetylene 0.1% (vol/vol) was added to a septum-capped bottle and was present throughout the incubation time as acetylene was still detectable in the headspace at the end of the incubation (data not shown). Acetylene was purified by passage through 5N H_2SO_4 , 5N NaOH, and water (Hyman and Arp, 1987). Both bottles were incubated for 3 weeks at 25°C in the dark. This assay was done in triplicate for each soil. At the end of incubation, 1 M KCl-extractable NH_4^+ -N and NO_3^- -N concentrations, and pH_{KCl} were determined. This approach was chosen to indicate the *in situ* N transformation rates.

Concentrations of extractable NO_3^- -N in soil at time zero and after 3 weeks were used to calculate net nitrification rate. The net ammonification rate and net N mineralization rate were calculated in the same manner; subtracting initial concentrations of NH_4^+ -N and $(\text{NH}_4^+ + \text{NO}_3^-)$ -N, respectively, from those measured at the end of the incubation. All N transformations were expressed on dry weight basis. The percent contribution of net ammonification and nitrification rates to net mineralization rate were calculated as net ammonification and net nitrification rates divided by net mineralization rate, respectively.

Table 1. Soil characteristics of the nine sites studied. Means of 3 replicates per site are presented (with SD).

Site name ^a	Latitude,Longitude	Total C ^b (%)	Total N ^b (%)	C/N ratio	Calcium ($\mu\text{mol g}^{-1}$ dw) ^c	pH (KCl)	NH ₄ ⁺ -N ($\mu\text{g g}^{-1}$ dw)	NO ₃ ⁻ -N ($\mu\text{g g}^{-1}$ dw)	N deposition (kg N ha ⁻¹ yr ⁻¹)	Presence of <i>Nitrospira</i> cluster 2
Nastola	60°56'N, 26°53'E	38.6 (0.11)	1.2 (0.00)	32.3 (0.02)	60.9 (1.50)	3.2 (0.01)	5.1 (0.26)	0.3 (0.08)	<1	No
Bentveld	52°22'N, 4°34'E	42.6 (0.16)	1.6 (0.00)	26.0 (0.05)	68.0 (0.02)	3.4 (0.08)	48.3 (0.47)	0.4 (0.06)	14-21	No
Appelscha	53°05'N, 6°40'E	47.8 (0.08)	1.8 (0.01)	27.1 (0.15)	23.6 (1.78)	2.9 (0.02)	28.5 (0.25)	0.4 (0.03)	21-35	No
Schoorl	52°43'N, 4°40'E	49.4 (0.03)	1.6 (0.00)	31.5 (0.06)	44.9 (0.27)	2.9 (0.01)	38.5 (0.19)	0.7 (0.03)	14-21	No
Hulshorst	52°21'N, 5°44'E	44.2 (0.23)	1.8 (0.00)	24.5 (0.11)	30.4 (0.92)	3.0 (0.02)	26.7 (0.30)	12.8 (0.20)	35-49	Yes
Roggebotzand	52°03'N, 5°50'E	43.2 (0.06)	1.7 (0.00)	25.4 (0.06)	96.0 (1.29)	3.4 (0.06)	28.4 (0.22)	14.7 (0.11)	21-35	Yes
Asbak	52°23'N, 4°34'E	43.1 (0.07)	1.7 (0.00)	25.0 (0.08)	76.3 (1.93)	3.4 (0.02)	38.5 (0.22)	15.9 (0.22)	14-21	Yes
Ysselsteyn	51°30'N, 5°55'E	45.3 (0.01)	2.2 (0.01)	20.3 (0.05)	35.6 (2.08)	2.9 (0.02)	29.1 (0.89)	17.7 (0.32)	>49	Yes
Wekerom	52°06'N, 5°41'E	44.5 (0.36)	1.8 (0.01)	24.9 (0.10)	27.7 (0.04)	2.9 (0.04)	39.6 (0.45)	21.2 (0.24)	35-49	Yes

^a Sites were ranked based on NO₃⁻-N initial concentration.

^b Percentage of total N and total C expressed on a dry soil basis.

^c dw = dry weight.

DNA extraction from soil and nested PCR. DNA was extracted from approximately 0.3 g sub-samples of soil (at field moisture content) using the FastDNA[®] SPIN Kit for Soil (Qbiogene, Carlsbad, CA, USA). The extracted DNA was cleaned three times with the Wizard DNA clean-up system (Promega, Madison, WI, USA). Independent DNA extractions were done in triplicate. A nested PCR was then carried out for all samples. Universal bacterial-specific 16S rDNA directed primers pAf/pHr (Edwards et al., 1989) were used in the primary amplification. PCR products were amplified from each DNA preparation in 50 µl reactions, 400 nM pAf/pHr, 0.2 mM dNTPs, 10 µg BSA, *Taq* DNA polymerase (2.5 unit, Promega), the buffer conditions recommended by the manufacturer, and 5 µl of each soil DNA extraction as template source. Reactions were carried out in a T3 thermocycler (Whatman Biometra[®]). The PCR thermocycling regime was as follows: 3 min at 95°C, followed by 30 cycles consisting of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C and a final cycle consisting of 10 min at 72°C. The PCR products were diluted 100 times and used as template in the second amplification using AOB specific primers, CTO189f-GC/CTO654r (Kowalchuk et al., 1997). The secondary amplifications used the same reaction volume, reagent concentrations and buffer conditions as described for the primer set of pAf/pHr. The PCR amplifications were performed at 95°C for 3 min after which 30 cycles were performed at 95°C for 1 min, 57°C for 1 min, 72°C for 1 min and a final cycle consisting of 10 min at 72°C. PCR products were examined by agarose gel electrophoresis.

TTGE analysis, cloning and sequencing. The PCR products were then profiled on TTGE (BIO-RAD Dcode[™] systems, Hercules, California, USA) as described by Laverman et al (2001). Gels (8% polyacrylamide, 1×TAE, 37.5:1 acrylamide:bisacrylamide, 8 M ureum) were run for 16 h at 60 V over temperature range 59.6°C-64.4°C, increasing at 0.3°C h⁻¹. Laverman et al. (2001) have compared TTGE and DGGE profiling of AOB and found that TTGE banding patterns are similar to those produced by DGGE. TTGE gels were stained with ethidium bromide, washed in distilled water and digitally recorded using a digital camera. To optimise the sensitivity of DNA detection, we also stained TTGE gel using SYBR gold, one of the most sensitive dyes, and the results were identical to those used ethidium bromide stain (data not shown). Independent DNA extractions and PCRs on the same soil sample revealed similar TTGE patterns (data not shown).

To determine the nucleotide sequence of TTGE bands, the centre of the band was cut out and incubated over-night in 50 µl TE at 4°C. This solution was use for PCR with primers CTO189f/CTO654r. The products were cleaned using Wizard PCR preps (Promega, Madison, WI, USA), ligated to the pGEM-T vector, and transformed into *Escherichia coli* JM109 competent cells (Promega, Madison, WI, USA) as specified by manufacturer. Transformed colonies were screened for inserts of the correct size by PCR amplification with CTO primer set. The selected clones were amplified with vector primer M13f (Promega, Madison, WI, USA) and analysed on an ALF Express II (Amersham Pharmacia Biotech.) according to the manufacturer's instruction. These selected clones were tested for correspondence with the original TTGE bands to make sure that the sequences were the target bands.

Data analysis. Non-parametric tests were used as homogeneity of variances of the data was not ensured. Mann-Whitney U tests were used to analyse differences in environmental factors and N transformation rates between two groups of sites, one group containing all sites that showed the presence of a particular 16S rRNA sequence, the other group containing the sites that did not show this sequence. Correlations between net nitrification rate, net ammonification rate and environmental factors were determined using Spearman's correlations. All statistical analyses were done using SPSS 11.5 for Windows.

Recovered 16S rDNA sequences were compared to sequences deposited in the GenBank DNA database by using BLAST algorithm (Altschul et al., 1997). Sequence alignments were performed using ClustalW Multiple sequence alignment program version 1.8 (<http://clustalw.genome.jp>). Only nucleotide positions that were unambiguously aligned were used in the subsequent phylogenetic analysis. Distance analysis of 427 nucleotide positions of the alignment was performed with Treecon version 1.3b software (van de Peer and de Wachter, 1994). Gaps were not taken into account in the analysis. The bootstrap analysis was based on 100 replicates.

Nucleotide sequence accession numbers. The nucleotide sequences have been deposited in GenBank under accession numbers AY683621 to AY683629.

Results

Study sites properties. Table 1 shows the soil characteristics of all study sites. The chosen sites show a large range in initial NH_4^+ -N, NO_3^- -N and Ca concentrations, total C, total N and pH. NH_4^+ -N was the predominant form of inorganic-N, and NO_3^- -N concentrations were very low in Schoorl, Nastola, Bentveld and Appelscha, accounting for less than 6% of inorganic N. In the other sites NO_3^- -N concentrations were much greater, accounting for 29-38% of inorganic N. Comparisons across all sites revealed that soil from Nastola has the lowest values of the initial NH_4^+ -N and NO_3^- -N contents, total C and total N compared to other soils, while other sites have shown no consistent trends concerning within site soil properties.

N transformation rates. The N transformation rates in nine forest soils after 3 weeks incubation are shown in Figure 1. At all sites, acetylene treatment resulted in a complete inhibition of net nitrification rate, suggesting that autotrophic nitrification was the dominant process. Concerning the net nitrification rate (Fig. 1a), the rates in Schoorl, Nastola, Bentveld and Appelscha were relatively low, less than $0.5 \mu\text{g g}^{-1}$ dry soil wk^{-1} . The rates in Ysselsteyn, Hulshorst and Wekerom were considerable higher at 11.0 - $14.4 \mu\text{g g}^{-1}$ dry soil wk^{-1} . The highest rate comprises soils from Roggebotzand and Asbak (29.4 - $31.6 \mu\text{g g}^{-1}$ dry soil wk^{-1}).

Net ammonification rates differed between sites (Fig. 1b). The lowest net ammonification rates were in Roggebotzand and Asbak (2.6 - $3.7 \mu\text{g g}^{-1}$ dry soil wk^{-1}). Nastola has a higher rate ($8.0 \mu\text{g g}^{-1}$ dry soil wk^{-1}) than Roggebotzand and Asbak, while Schoorl, Ysselsteyn, Hulshorst and Wekerom have higher (a factor three) rates than

Nastola. The highest rates were found in Bentveld and Appelscha (35.0-37.3 $\mu\text{g g}^{-1}$ dry soil wk^{-1}).

Furthermore, net mineralization rates in all soils were comparable with the exception of soils from Schoorl and Nastola (Fig 1c). The net mineralization rates in Schoorl and Nastola were lower than other in sites, 22.1 and 8.2 $\mu\text{g g}^{-1}$ dry soil wk^{-1} , respectively. Moreover, the percent contribution of ammonification and nitrification to net mineralization rates in Schoorl, Nastola, Bentveld and Appelscha are around 99% and 1%, respectively. The percent contribution of these two processes to net mineralization rates in Ysselsteyn, Hulshorst and Wekerom are around 64% and 36%, respectively, and in Roggebotzand and Asbak around 9% and 91%, respectively.

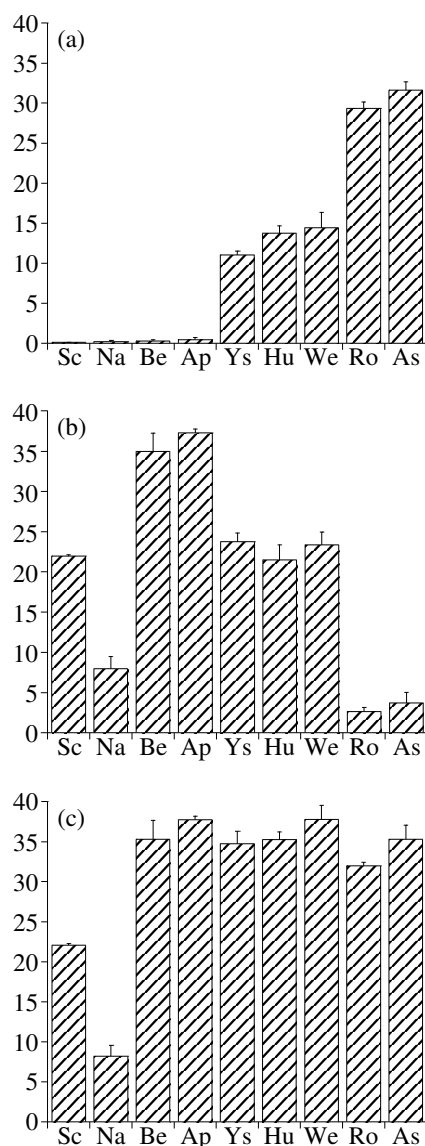


Figure 1. Net nitrification rate (a), net ammonification rate (b), and net mineralisation rate (c) in Scots pine forest soils. All rate units in $\mu\text{g g}^{-1}$ dry soil wk^{-1} . Error bars are standard deviations. The abbreviations on the x axis represent the following sites: Sc, Schoorl; Na, Nastola; Be, Bentveld; Ap, Appelscha; Ys, Ysselsteyn; Hu, Hulshorst; We, Wekerom; Ro, Roggebotzand; As, Asbak.

Correlation between soil properties and N transformation rates. Table 2 shows Spearman's correlations between different soil properties. Positive correlations were found between pH and Ca content, initial NO_3^- -N concentration and total N, N deposition rate and total N, and N deposition rate and initial NO_3^- -N concentration. Negative correlations were found between C/N ratio and total N, Ca content and total C, Ca content and total N, pH and total C, initial NO_3^- -N concentration and C/N ratio, and N deposition rate and C/N ratio.

Table 2. Spearman's correlation coefficients between net nitrification, ammonification, and mineralisation rates and soil properties among sites (only significant correlations are shown).

	Total C	Total N	C/N	Ca	pH	NO_3^- -N	NH_4^+ -N	Ndep.	NNR	NAR
Total N										
C/N		-0.895**								
Ca	-0.608**	-0.525*								
pH	-0.713**			0.748**						
NO_3^- -N		0.670**	-0.761**							
NH_4^+ -N										
N dep. ^a		0.841**	-0.738**			0.603**				
NNR ^b		0.482*	-0.612**			0.680**		0.419*		
NAR ^c				-0.674**	-0.401*				-0.455*	
NMR ^d		0.682**	-0.484*	-0.600**			-0.407*	0.494**		0.615**

^a Ndep. = N deposition rate

^b NNR = Net nitrification rate

^c NAR = Net ammonification rate

^d NMR = Net mineralisation rate

* $P \leq 0.05$

** $P \leq 0.01$

A weak negative correlation was observed between net ammonification rate and net nitrification rate. Net mineralisation was positively correlated to net ammonification rate. Moreover, these rates were also correlated to some soil properties (Table 2). Net nitrification rate was negatively correlated to C/N ratio, and positively correlated to total N, initial NO_3^- -N concentration and N deposition rate. Net ammonification rate was negatively correlated to Ca content and pH. Net mineralisation rate was positively correlated to total N and N deposition rate, and negatively correlated to C/N ratio, Ca content and initial NH_4^+ -N concentration.

β -subdivision AOB-like 16S rDNA. Figure 2 shows that very similar AOB TTGE banding patterns were recovered for soil samples from Ysselsteyn, Hulshorst, Wekerom, Roggebotzand and Asbak (Fig. 2). These doublet bands co-migrated with products from cloned standards representing *Nitrosospira* clusters 2 and 3. The TTGE bands apparent for the Schoorl, Nastola, Bentveld and Appelscha samples did not clearly co-migrate with any of the cloned standards (Fig. 2). TTGE patterns of PCR products from independent PCRs on the same sample and from independent DNA extractions showed no variation in the number of bands and migration position (results not shown). These findings confirmed the reproducibility of the PCR-TTGE method. In

both reference clone and environmental TTGE patterns, bands often occurred in doublets, which is consistent with previous results which showed that a single template sequence can give rise to multiple TTGE bands due to an ambiguous position in the CTO reverse primer (Kowalchuk et al., 1997).

Co-migration of bands from *Nitrosospira* clusters 2 and 3 and the lack of co-migration for bands from four samples with any of the standards prevented unambiguous identification of AOB clusters based on migration pattern alone. It was therefore necessary to excise and reamplify TTGE bands for cloning, sequencing and phylogenetic analysis.

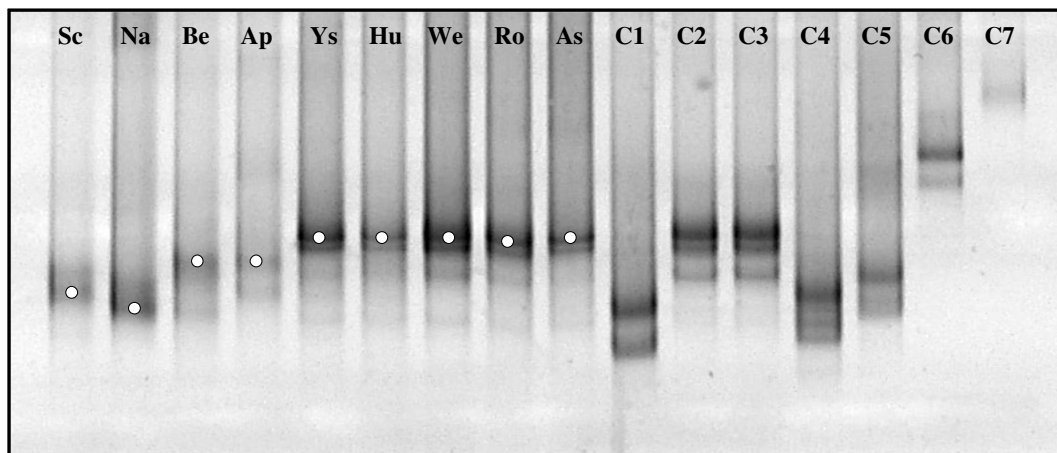


Figure 2. PCR-TTGE analysis of ammonia-oxidising bacteria like 16S rDNA fragment from Scots pine forest soils. The abbreviations represent the following sites and reference clones: Sc, Schoorl; Na, Nastola; Be, Bentveld; Ap, Appelscha; Ys, Ysselsteyn; Hu, Hulshorst; We, Wekerom; Ro, Roggebotzand; As, Asbak; C1-C4, reference clones for *Nitrosospira* cluster 1-cluster 4; C5-C7, reference clones for *Nitrosomonas* cluster 5-cluster 7. Sequenced bands are indicated in the figure with white circles.

Phylogenetic analysis of the recovered sequences derived from Ysselsteyn, Hulshorst, Wekerom, Roggebotzand and Asbak bands placed these sequences (GenBank accession nos. AY683625, AY683626, AY683627, AY683628 and AY683629, respectively) in *Nitrosospira* cluster 2 (Fig. 3), which is in agreement with the TTGE analysis. The similarity between these five sequences ranged from 98% to 100%. The sequences showed the closest affinity to *Nitrosospira* sp. strains III7 and AHB1. The similarity to these known β -subdivision AOB sequences ranged from 95% to 99%. Phylogenetic analysis of Schoorl, Nastola, Bentveld and Appelscha bands placed their recovered sequences (GenBank accession nos. AY683621, AY683622, AY683623, and AY683624, respectively) outside the *Nitrosospira/Nitrosomonas* clade. Statistical analysis based on non-parametric analysis of variance (Mann-Whitney U test) on soils with and without recovered *Nitrosospira* cluster 2-like 16S rDNA sequences revealed that soils with recovered *Nitrosospira* cluster 2-like 16S rDNA sequences were significantly higher in total N, initial NO_3^- -N concentration and atmospheric N deposition, while C/N ratio was lower in these soils. Net nitrification rate was higher in these soils, while net ammonification rate was lower in these soils.

Discussion

In this study, we found that environmental factors, such as C/N ratio of the F layer and atmospheric N deposition affect the presence of the AOB, which may explain the big differences in N transformation rates between the different forest soils. The presence of AOB was determined by cultivation-independent, molecular methods. The different steps (DNA extraction, PCR, and profiling) in such a molecular approach have their pitfalls (von Wintzingerode et al., 1997). However, since all samples were treated similarly, these pitfalls can be considered the same for all samples, allowing between-sample comparisons.

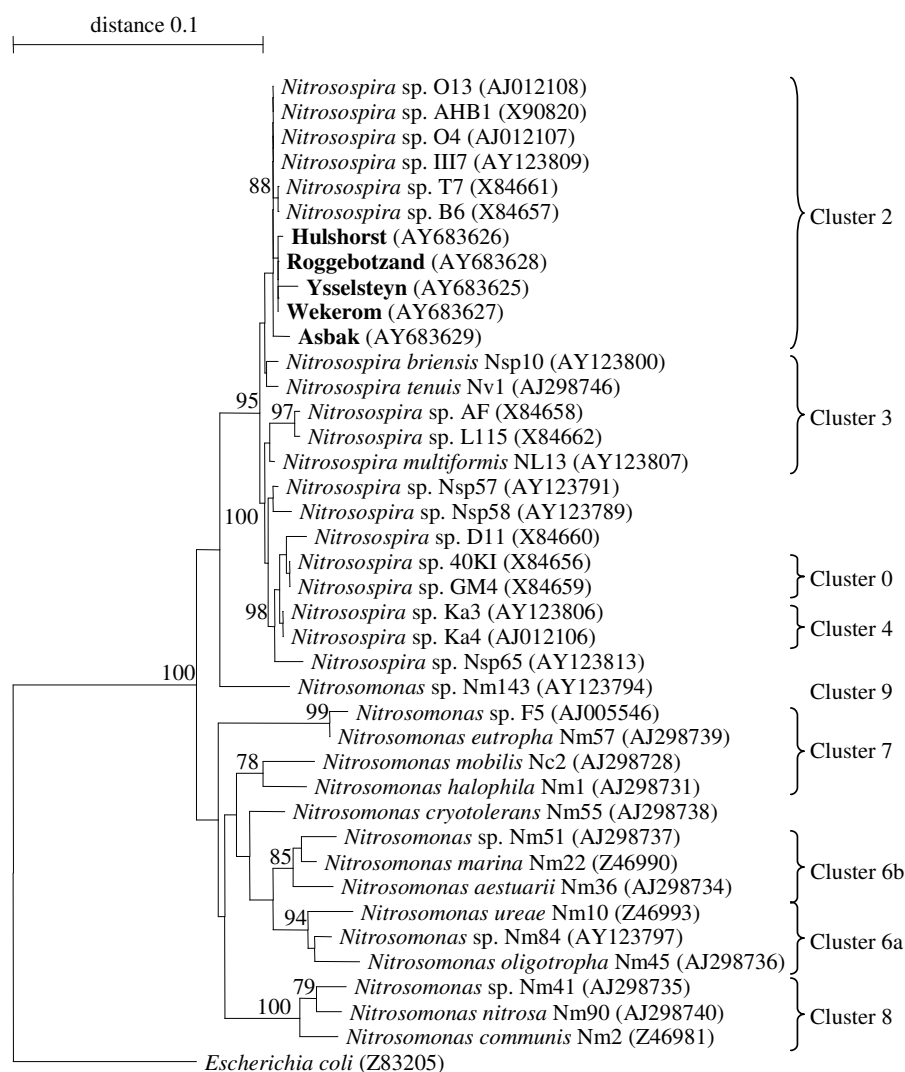


Figure 3. Neighbour-joining tree of partial 16S rDNA sequences (427 informative positions) recovered from five forest soil samples (in bold) using CTO primers, showing the similarity between the retrieved sequences and their placement in *Nitrosospira* cluster 2. Cluster designations are those used by Koops et al. (2003). Bootstrap values are given at nodes when they exceed 75% of replicates.

In soils with high net nitrification rates, we detected only *Nitrosospira* cluster 2-like sequences. In soils with low net nitrification rates, we could not detect any AOB-like

sequences even though a nested PCR approach was used. We found only non-AOB sequences in these soils. The detection of non-AOB-like sequences using the CTO primers was not surprising, since these primers have a relatively low specificity (Koops et al., 2003; Purkhold et al., 2000). Purkhold et al. (2000) demonstrated that none of the primers and probes targeting the 16S rRNA or the 16S rRNA-encoding gene of AOB can cover all cultured species along with being 100% specific for these AOB. The predominance of *Nitrospira* cluster 2-like sequences detected in this study is not likely to be due to high selectivity of the CTO primers. Kowalchuk et al. (1997) tested the selectivity in the CTO primers by performing on DNA templates containing different ratios of clones pH4.2A/6 (*Nitrospira* cluster 2) and pH4.2A/23 (*Nitrosomonas* cluster 6), followed by separation of these two clones in DGGE. The DGGE profiling revealed bands with relative intensities that were in good agreement with ratios between the two clones in the starting DNA template.

We also do not expect that the predominance of *Nitrospira* cluster 2-like sequences is due to a failure of FastDNA Spin Kit to extract DNA of several types of highly abundant AOB. *Nitrospira* clusters 3 (Mintie et al., 2003) and 4 (Bäckman et al., 2004; Mintie et al., 2003) and *Nitrospira multiformis* and *Nitrosomonas europaea* (Briones et al., 2002) could also be detected in soil systems using the FastDNA Spin Kit. Furthermore, Laverman et al. (2001) previously used a modified mechanistic cell disruption method to extract DNA from two forest soils, Wekerom and Roggebotzand, that were also addressed in this study and also detected *Nitrospira* cluster 2, exclusively, based on 16S rRNA gene based analysis. Moreover, Laverman et al. (2001) confirmed the dominance by members of *Nitrospira* cluster 2 by using a set of primers that target the ammonia monooxygenase (*amoA*) gene. They found that all AOB populations detected in Wekerom and Roggebotzand soils were closest related to a cultured representative of *Nitrospira* cluster 2, strain *Nitrospira* sp. AHB1.

Nevertheless, we used only one non-quantitative analysis for detection of AOB sequences. Since no single technique can be expected to provide a comprehensive view of microbial populations in their environment (Kowalchuk and Stephen, 2001), we might have overlooked other AOB populations possibly present. Therefore, we cannot rule out that additional AOB populations will be found if a combination of different techniques and molecular markers would be applied on all samples.

Soils with high nitrification rates are characterised by high initial NO_3^- -N concentrations, low C/N ratios (or high total N) and high atmospheric N deposition. According to the atmospheric N deposition map of The Netherlands (RIVM, 2001), sites with high nitrification rates can be classified as sites with intermediate to high atmospheric N deposition. Thus, our observations are in agreement with earlier findings (Bengtsson et al., 2003; Gundersen et al., 1998a; 1998b; Laverman et al., 2002; Persson et al., 2000). As the soils with high nitrification rates were all characterised by a predominance of *Nitrospira* cluster 2-like sequences, the C/N values and the high atmospheric N deposition of these sites are probably favourable for the presence of *Nitrospira*. In acid coniferous forest soil profiles with low N deposition ($14.8 \text{ kg N ha}^{-1} \text{ yr}^{-1}$) at a site in South Western Sweden, AOB-like sequences could not be detected either (Bäckman et al., 2003).

Net nitrification rates and the presence of *Nitrospira* cluster 2 were not correlated to soil pH. This may be due to the relatively small range of soil pH values and small

number of study sites, although it has been observed in acid forest soil that *Nitrospira* cluster 2 is present regardless of soil pH (Bäckman et al., 2004).

Since we did not detect any other cluster types, this study suggests the dominance of a single 16S rDNA sequence type within natural AOB communities in geographically distant acid Scots pine forest soils. So far, similar little variation of AOB community composition, with the exclusive detection of a single *Nitrospira* 16S rDNA sequence cluster, was found in a nitrogen-saturated coniferous forest soil (Laverman et al., 2001). These results are in contrast with other molecular studies of AOB in soil habitats where larger 16S rDNA sequence diversity has been detected (e.g. Bäckman et al., 2004; Bruns et al., 1999; Kowalchuk et al., 1997; Kowalchuk et al., 2000b; Stephen et al., 1996). Environmental factors in our study sites probably affect the activity and presence, but not the cluster type of AOB.

The AOB found in this study at Wekerom is similar to the AOB previously detected at this site by Laverman et al. (2001). They also found little variation between different organic layers and throughout the year in this site. We suggest that this high degree of spatio-temporal stability of AOB in acid Scots pine forest soils is a general phenomenon.

In general, it appears that members of *Nitrospira* cluster 2 dominate in acidic soils with high nitrification rates [this study, Stephen et al., 1996; Kowalchuk et al., 2000b; Laverman et al., 2001). However, *Nitrosomonas europaea*-like populations dominating in acidic Belgian forest soils (Carnol et al., 2002) show that this is not always the case. Which and how environmental factors select for certain sequence cluster(s) of AOB remains an enigma. The correlation of N transformation rates to C/N ratio (or total N), atmospheric N deposition and the presence of AOB suggests that N transformation rates are not simply the result of the effects of individual ecosystem properties, but are driven by the interactions between these properties.

Acknowledgements

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CHAPTER 3

NET NITRIFICATION RATE AND PRESENCE OF *NITROSOSPIRA* CLUSTER 2 IN ACID CONIFEROUS FOREST SOILS APPEAR TO BE TREE SPECIES SPECIFIC

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Abstract

The impact of four coniferous tree species and their corresponding soil factors on N transformation rates and presence of ammonia-oxidising bacteria (AOB) was studied in a forest at Appelscha, The Netherlands. We focussed on fragmented (F) soil layers of Scots pine (*Pinus sylvestris* L.), Norway spruce (*Picea abies* (L.) Karst.), Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco) and European larch (*Larix decidua* Miller). The presence of AOB was studied by denaturing gel electrophoresis analysis of the 16S rRNA and *amoA* genes, amplified by PCR, with subsequent band excision, cloning and nucleotide sequence analysis. Scots pine soil had relatively low net nitrification rate, while the three other soils showed high net nitrification rates. These net nitrification rates negatively correlated to net ammonification rates and to C/N ratios and positively correlated to total N and Ca content, whereas the net ammonification rate was negatively correlated to total N and Ca content. All 16S rRNA and *amoA* sequences found in soils with high nitrification rates belonged to *Nitrosospora* cluster 2 and were highly similar (99-100% similarity). We conclude that tree species, possibly through their effect on soil C/N ratios, determines the presence of *Nitrosospora* cluster 2. Whenever AOB are present, however, the AOB community composition appears to be similar.

Keywords: coniferous tree species; C/N ratios; N transformation rates; ammonia-oxidising bacteria; *Nitrosospora* cluster 2

Introduction

Since tree species have a great impact on soil properties (Binkley and Giardina, 1998; Knops et al., 2002; Myers et al., 2001; Northup et al., 1998; Stark and Firestone, 1996), they can determine associated soil microbial biomass, activity and community structure (Bauhus et al., 1998; Côte et al., 2000; Priha and Smolander, 1999; Priha et al., 2001; Smolander and Kitunen, 2002; Templer et al., 2003). This can be explained by the differences in leaf litter quality across stands of different tree species (Pastor and Post, 1986). It has been shown that microbial activities and microbial biomass correlate to the quality and quantity of soil organic matter under Norway spruce (Merilä and Ohtonen, 1997). Significant differences in microbial activity were found in the upper soil horizons under pine, spruce and birch (Priha and Smolander, 1999). Plant species-induced effects on bacterial community structures in soil were also demonstrated by cultivation-independent, 16S rRNA gene based fingerprinting using denaturing gradient gel electrophoresis (DGGE) (Kowalchuk et al., 2002). Furthermore, under oak species microbial communities were dominated by fungi, while communities under sugar maple-basswood stands were dominated by bacteria (Myers et al., 2001).

The variation in N transformations across forest soils appears also to be related to the type of tree species (e.g. Chen and Stark, 2000; Finzi et al., 1998; Lovett et al., 2004; Menyailo et al., 2003; Priha and Smolander, 1999). As the nitrification process (the conversion of ammonium to nitrate via nitrite) is critical to the N transformations in forest soils, the presence, type and activity of ammonia-oxidising bacteria (AOB) are likely to be influenced by tree species.

The aim of the present study was to elucidate the relationship between soil factors, N transformation rates and the presence of AOB in soils under different coniferous tree species, growing at short distances from each other. We studied the N transformation rates and the presence of AOB, by 16S rRNA and *amoA* gene analysis, from four soils under different types of coniferous trees in a forest area with low N deposition (Appelscha, The Netherlands). The tree species were of the same age, growing in the same soil type, at the same elevation and under identical climatic conditions, and receiving a similar relatively low, according Dutch standards, N deposition (14-21 kg ha⁻¹ y⁻¹ (RIVM, 2002)). The differences between the soils are therefore related to the different tree species.

Materials and Methods

Field site and forest floor collection. The forest floor layer utilized in this study was collected from different forest soils at Appelscha (53°05'N latitude, 6°40'E longitude), The Netherlands (Fig. 1). Scots pine (*Pinus sylvestris* L.), Norway spruce (*Picea abies* (L.) Karst.), Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco) and European larch (*Larix decidua* Mill.) stands were selected, representing the major coniferous forest types found in this area. At each sampling site, ten samples (15×20 cm) of the forest floor (F layer) were randomly collected from a 5×5 m area, then pooled in a clean plastic bag and returned to the laboratory, as described in Chapter 2. The field-moist soils were immediately passed through a 4 mm sieve in the laboratory and homogenised

by hand. Sieving through a 2 mm sieve has hardly any effect on mineralisation (Kristensen et al., 2003). Sub-samples of 30 g of the sieved material were stored at -20°C until molecular analysis. The remaining sieved material was kept at 5°C , to minimise changes in initial conditions across the soils, for less than a week until further analysis.

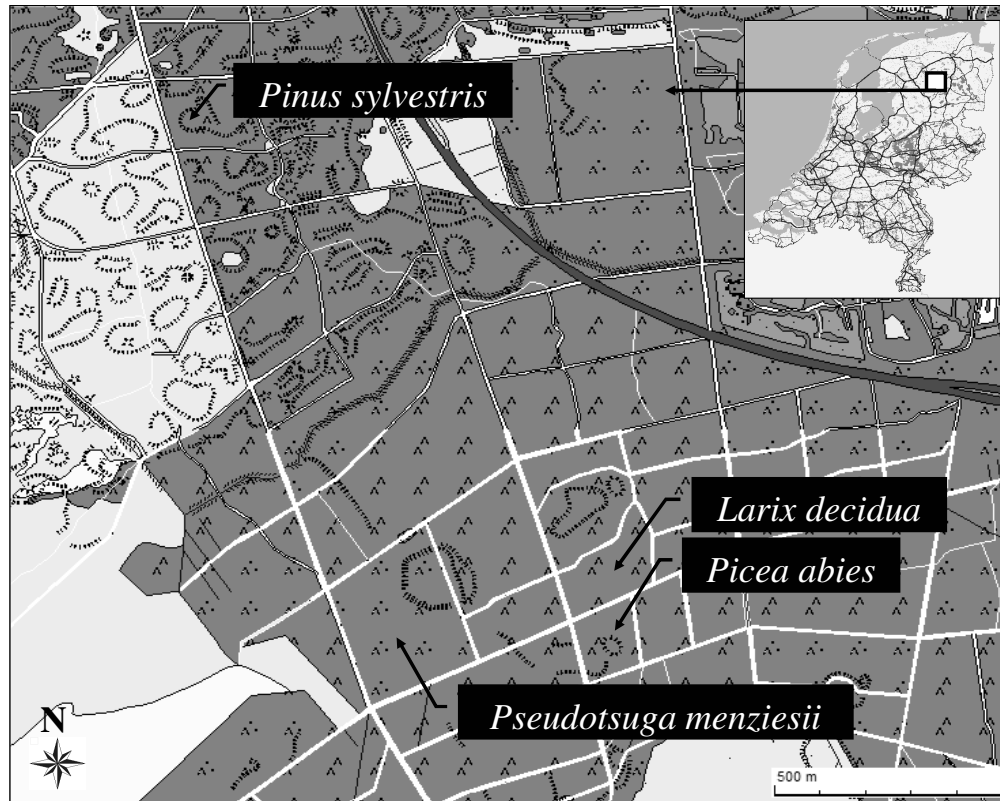


Figure 1. Location of four different tree species in Appelscha, The Netherlands.

Soil characteristics. Sub-samples of the sieved material were analysed for total N and total C on a Carlo Erba Strumentazione elemental analyser (model 1106), and Ca content on an atomic absorption spectrophotometer (AAS) (Perkin Elmer, model 1100B). Moisture contents of the soils were determined by drying fresh samples at 50°C for 3 days. Extractable NH_4^+ -N and NO_3^- -N concentrations were determined by extraction of the samples in 1 M KCl (15 g field-moist soil:100 ml 1 M KCl). After filtration, the extract was analysed for NH_4^+ -N and NO_3^- -N concentrations and pH_{KCl} .

Enumeration of AOB. AOB were enumerated applying a most probable number (MPN) technique using microtitre plates (Rowe et al., 1977). For each of the four soil samples, three soil suspensions were prepared by mixing 1 g soil in 9 ml sterile phosphate buffer. The soil suspensions were shaken for 2 h on a shaker (100 rev min^{-1}) at room temperature. The soil suspension was allowed to settle for 15 min, before the supernatant containing the desorbed bacterial cells was decanted into 2 ml sterile eppendorf. The wells were filled with 200 μl of the ammonium-calcium carbonate

medium (Alexander and Clark, 1965) and 50 µl soil suspension was added to the first row of eight wells, mixed well, and 50 µl transferred to the next row. This process is continued until serial dilutions have been carried out across the plate. The result is 12 fivefold serial dilutions with eight replicates at each dilution. The plates were wrapped in parafilm to reduce evaporation and incubated in the dark at 22°C for two months. The presence of nitrate and nitrite was checked as an indicator of ammonia oxidation by the addition of diphenylamine reagent (0.2 g in 100 ml concentrated H₂SO₄). The numbers were estimated by MPN table constructed for the microtitre plate method (Rowe et al., 1977).

N transformation rates determination. N transformation rates were determined using the method described by Laverman et al. (2000). Sub-samples for incubation were brought to 65% moisture content by adding demineralised water. Two 250 ml screw-cap bottles were filled with 15 g fresh material each. One bottle was capped with a regular cap and the other with a septum-contained cap. Acetylene 0.1% (vol/vol) was added to a septum-capped bottle and was present throughout the incubation time as acetylene was still detectable in the headspace at the end of the incubation (data not shown). Acetylene was purified by passage through 5N H₂SO₄, 5N NaOH, and water (Hyman and Arp, 1987). Both bottles were incubated for 3 weeks at 25°C in the dark. This assay was done in triplicate for each soil. At the end of incubation, 1 M KCl-extractable NH₄⁺-N and NO₃⁻-N concentrations, and pH_{KCl} were determined.

Concentrations of extractable NO₃⁻-N in soil at time zero and after 3 weeks were used to calculate net nitrification rate. The net ammonification rate and net N mineralization rate were calculated in the same manner; subtracting initial concentrations of NH₄⁺-N and (NH₄⁺+NO₃⁻)-N, respectively, from those measured at the end of the incubation. All N transformations were expressed on dry weight basis.

PCR of 16S rRNA and amoA genes. DNA was extracted from approximately 0.3 g sub-samples of soil (at field moisture content) using the FastDNA[®] SPIN Kit for Soil (Qbiogene, Carlsbad, CA, USA). The extracted DNA was cleaned three times with the Wizard DNA clean-up system (Promega, Madison, WI, USA). DNA extractions were done in duplicate.

All 50 µl reaction mixtures contained 400 nM of each primer, 0.2 mM dNTPs, 10 µg BSA, Taq DNA polymerase (2.5 unit, Promega), the buffer conditions recommended by the manufacturer and 5 µl template DNA. A nested PCR was carried out for all samples. Universal bacterial-specific 16S rRNA directed primers pAf/pHr (Edwards et al., 1989) were used in the primary amplification aimed at detecting AOB 16S rRNA genes. The PCR thermocycling regime was: 3 min at 95°C, followed by 30 cycles consisting of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C and a final cycle consisting of 10 min at 72°C. The PCR products were used as template in the second amplification using AOB specific primers, CTO189f-GC/CTO654r (Kowalchuk et al., 1997). The PCR thermocycling regime was: 3 min at 95°C, followed by 30 cycles consisting of 1 min at 95°C, 1 min at 57°C, and 1 min at 72°C and a final cycle consisting of 10 min at 72°C. PCR products were examined by agarose gel electrophoresis.

In addition to the 16S rRNA analysis, the AOB primer set (amoA-1F/amoA-2R-TC) targeting the ammonia monooxygenase subunit A gene (*amoA*), described by Nicolaisen and Ramsing (2002), was used for comparison. The PCR thermocycling regime was: 5 min at 93°C, followed by 35 cycles consisting of 30 sec at 93°C, 45 sec at 57°C, and 45 sec at 72°C and a final cycle consisting of 5 min at 72°C. Reactions were carried out in a T3 thermocycler (Whatman Biometra®). The PCR products were used as template in the second amplification using amoA-1F-Clamp/amoA-2R-TC (Nicolaisen and Ramsing, 2002). The secondary amplifications were done with PCR conditions described above, but run for 18 cycles. PCR products were examined as above.

TTGE and DGGE analyses. 16S rRNA gene fragments were profiled by temporal temperature gradient electrophoresis (TTGE, BIO-RAD Dcode™ systems, Hercules, California, USA) as described by Laverman et al. (2001). Gels (8% polyacrylamide, 1×TAE, 37.5:1 acrylamide:bisacrylamide, 8 M ureum) were run for 16 h at 60 V over temperature range 59.6°C-64.4°C, increasing at 0.3°C h⁻¹. Laverman et al. (2001) have compared TTGE and DGGE profiling of AOB and found that TTGE banding patterns are similar to those produced by DGGE.

amoA gene fragments were profiled by DGGE as described by Nicolaisen and Ramsing (2002) with a gradient of 30-70% denaturant. Gels were run for 15 h at 100 V in 1×TAE buffer at a constant temperature of 60°C. TTGE and DGGE gels were stained with SYBR gold (Molecular Probes) and photographed on a UV transilluminator with a digital camera.

Cloning and sequencing. The centre of the TTGE and DGGE bands from two independent electrophoresis profiles per sample were excised using a sterile pipet tip, incubated over-night in 50 µl TE at 4°C and used for PCR with primers CTO189f/CTO654r or amoA-1F/amoA-2R-TC, respectively. The products were cleaned using Wizard PCR preps (Promega, Madison, WI, USA), ligated to the pGEM-T vector, and transformed into Escherichia coli XL-1 Blue competent cells (Promega, Madison, WI, USA) as specified by the manufacturer. Clones were screened for inserts of the correct size by PCR amplification and one correctly sized clone, per independent electrophoresis profile and per sample, was sequenced using an ABI PRISM® 3100 Genetic Analyzer.

Data analysis. Non-parametric tests were used as homogeneity of variances of the data was not ensured. The Kruskal-Wallis tests were used to analyse differences in environmental factors and N transformation rates between soils under tree species. These tests were also used to analyse differences in environmental factors and N transformation rates between two groups of soils under tree species, one group containing soils under tree species that show the presence of a particular 16S rRNA and *amoA* sequences, the other group containing soils under tree species that did not show these sequences. Correlations between N transformation rates and environmental factors were determined using Spearman's correlations. All statistical analyses were done using SPSS 11.5 for Windows.

Sequence analyses were done using BLAST algorithm (Altschul et al., 1997). Sequence alignments were performed using ClustalW Multiple sequence alignment

program version 1.8 (<http://clustalw.genome.jp>). Distance analysis was performed with Treecon version 1.3b software (van de Peer and de Wachter, 1994). Gaps were not taken into account in the analysis. The bootstrap analysis was based on 1000 replicates.

Results

Soil properties and N transformation rates. Soil properties differed between soils under the four tree species (Table 1). Comparisons across all soils revealed that Scots pine soil had the lowest total N and initial NO_3^- -N concentration, and the highest C/N ratio. Norway spruce soil had the lowest pH and Ca concentration, and the highest total C and initial NH_4^+ -N concentration. Douglas fir soil had the lowest total C and C/N ratio, and the highest initial NO_3^- -N concentration. European larch soil had the lowest initial NH_4^+ -N concentration, and the highest pH, Ca and total N. NH_4^+ -N was the predominant form of inorganic-N, accounting for 65-98% of inorganic N.

Table 1. Soil characteristics of the forest soils studied. Values represent means of 3 replicates \pm SD.

Tree species	pH (KCl)	Calcium ($\mu\text{mol g}^{-1}$ dry soil)	Total C (%)	Total N (%)	C/N	NH_4^+ -N ($\mu\text{g g}^{-1}$ dry soil)	NO_3^- -N ($\mu\text{g g}^{-1}$ dry soil)	Density of AOB (MPN counts g^{-1} dry soil)
<i>P. sylvestris</i>	2.94 (0.02)	23.6 (1.78)	47.8 (0.08)	1.8 (0.01)	27.1 (0.15)	28.5 (0.25)	0.4 (0.03)	0
<i>P. abies</i>	2.87 (0.02)	22.8 (0.26)	48.6 (0.09)	2.2 (0.01)	22.2 (0.05)	106.1 (0.21)	14.8 (0.30)	405 (271)
<i>P. menziesii</i>	2.90 (0.02)	42.2 (0.23)	45.5 (0.26)	2.2 (0.02)	20.4 (0.06)	61.3 (0.90)	30.9 (0.76)	37 (64)
<i>L. decidua</i>	3.07 (0.01)	79.1 (1.58)	46.9 (0.20)	2.3 (0.02)	20.8 (0.05)	24.2 (0.24)	12.8 (0.26)	354 (313)

There were significant differences in the rates of net nitrification and ammonification between the soils (Fig. 2a and b), net nitrification rate was nearly zero in Scots pine soil, but much higher in Norway spruce, Douglas fir soils and European larch soils. Net ammonification rate decreased in the following sequence: Scots pine>Norway spruce>Douglas fir>European larch. Net N mineralisation rates were also different between different tree species (Fig. 2c), Scots pine soil had the highest rate and the other soils were statistically indistinguishable.

Table 2 shows Spearman's correlations between soil properties and N transformation rates. There were significant negative correlations between C/N ratio and total N, C/N ratio and initial NO_3^- -N concentration, pH and initial NO_3^- -N concentration, and pH and initial NH_4^+ -N concentration. Significant positive correlations were found between C/N ratio and total C, and Ca content and total N.

Net nitrification rate was negatively correlated to net ammonification rate, while net ammonification rate was positively correlated to net mineralisation rate. Net nitrification rate was negatively correlated to C/N ratio and positively correlated to total N and Ca content. Net ammonification rate was negatively correlated to total N and Ca content.

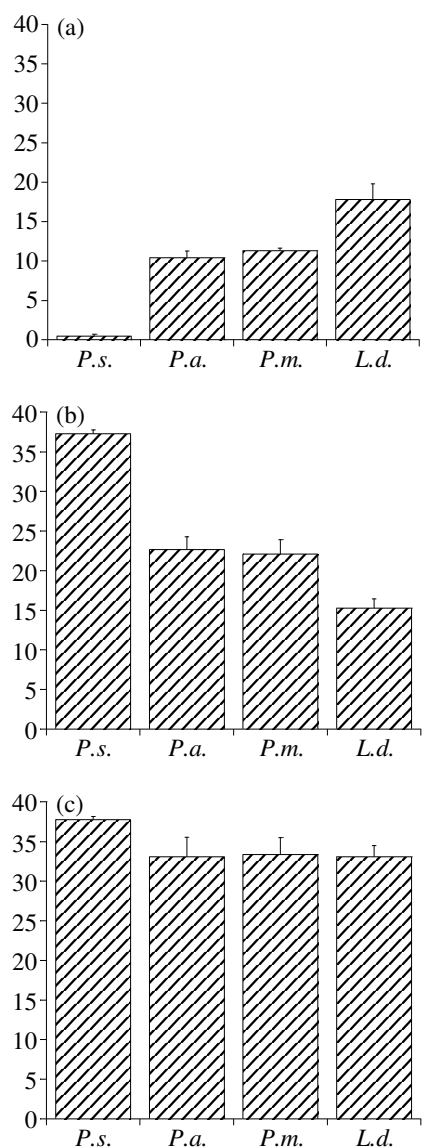


Figure 2. Net nitrification (a), net ammonification (b), and net mineralisation (c) rates in four tree species. All units are expressed as $\mu\text{g g}^{-1}$ dry weight week^{-1} . Error bars are standard deviations. The abbreviations in the x axis represent the following tree species: *P.s.*, *Pinus sylvestris*; *P.a.*, *Picea abies*; *P.m.*, *Pseudotsuga mensiezii*; *L.d.*, *Larix decidua*.

β -subdivision AOB-like community structure. The TTGE banding patterns for AOB 16S rRNA gene fragments from Norway spruce, Douglas fir and European larch soils were similar (Fig. 3a), indicating low diversity of the AOB community with only one discernible doublet band detected. PCR products from these soils co-migrated with products from cloned standards representing *Nitrospira* clusters 2 and 3. In both reference clone and environmental TTGE patterns, bands often occurred in doublets, which is consistent with previous results which showed that a single template sequence can give rise to multiple TTGE bands due to an ambiguous position in the CTO reverse primer (Kowalchuk et al., 1997). Phylogenetic analysis of the 16S rRNA gene fragments (Fig. 4) excised from the TTGE profiles of these soils assigned them (99-

100% similarity) to *Nitrosospira* cluster 2 and the sequences were closest related (99% similarity) to that of *Nitrosospira* sp. strains III7 and AHB1, isolated from acid soils.

In contrast, the band pattern from Scots pine soil was different from the other soils (Fig. 3a). The bands did not clearly co-migrate with any of the cloned AOB standards. Phylogenetic analysis of the 16S rRNA gene fragments excised from TTGE profiles of this soil placed their recovered sequences outside the *Nitrosospira*/*Nitrosomonas* clade (data not shown).

Table 2. Spearman's correlation coefficients between net N transformation rates and soil properties among tree species (only significant correlations are shown).

	Total C	Total N	C/N	Ca	pH	NO ₃ ⁻ -N	NH ₄ ⁺ -N	NNR	NAR
Total N									
C/N	0.738*	-0.881**							
Ca		0.833*							
pH									
NO ₃ ⁻ -N			-0.786*		-0.636*				
NH ₄ ⁺ -N					-0.914**				
NNR ^a		0.952**	-0.738*	0.810*					
NAR ^b		-0.810*		-0.714*				-0.846**	
NMR ^c									0.818**

^a Ndep. = N deposition rate

^b NNR = Net nitrification rate

^c NAR = Net ammonification rate

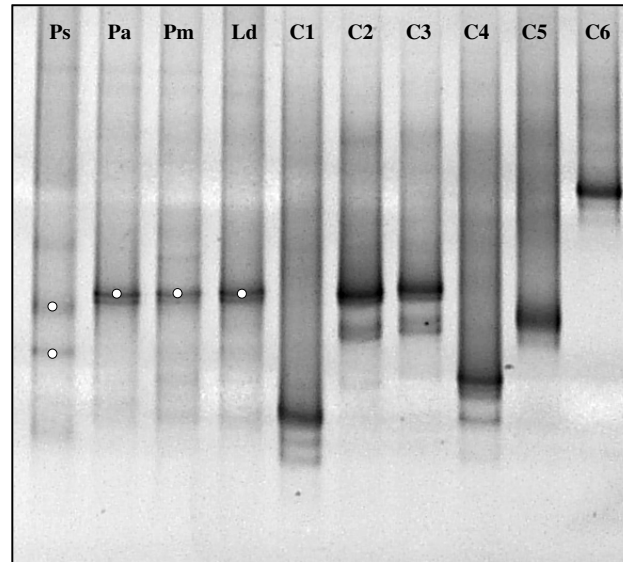
^d NMR = Net mineralisation rate

* $P \leq 0.05$

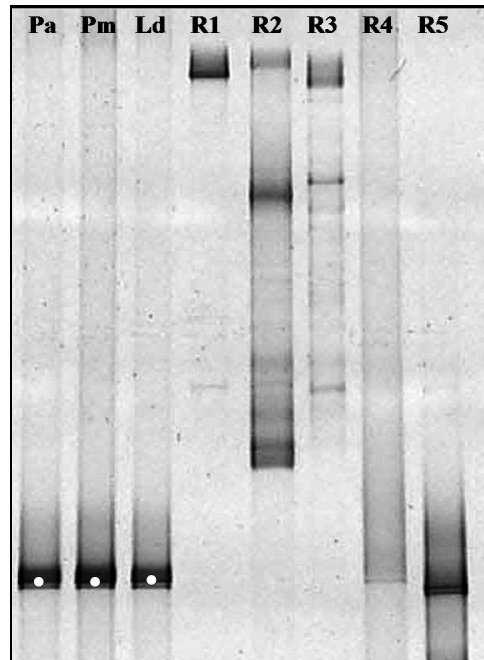
** $P \leq 0.01$

Results from amplifying, profiling and sequencing *amoA* genes confirmed the observations made based on analysis of 16S rRNA genes. Amplified *amoA* gene fragments from Norway spruce, Douglas fir and European larch soils migrated to similar end-positions in DGGE and also co-migrated with products from *amoA* cluster 2 (*Nitrosospira* sp. AHB1) and cluster 3 (*Nitrosospira briensis*) (Fig. 3b). Phylogenetic analysis of the *amoA* gene fragments (Fig. 5) excised from the DGGE profiles of these soils grouped them (99-100% similarity) within *Nitrosospira* cluster 2, in accordance with phylogenetic tree analysis of the 16S rRNA gene fragments. These *amoA* sequences were also closely related (97-100% similarity) to that of *Nitrosospira* sp. strains III7 and AHB1. On the other hand, Scots pine soil sample did not generate PCR product using the *amoA* primers even though a nested PCR was carried out for this sample.

Soils with recovered *Nitrosospira* cluster 2-like 16S rRNA and *amoA* sequences were significantly higher in total N and initial NO₃⁻-N concentration and lower in C/N ratio. Net nitrification rate was higher in these soils, while net ammonification and mineralisation rates were lower in these soils (Kruskal Wallis tests, $P < 0.05$).



(a)



(b)

Figure 3. PCR-TTGE analysis of AOB-like 16S rRNA fragment (a) and PCR-DGGE analysis of AOB-like *amoA* fragment (b) from four tree species soils. The abbreviations represent the following tree species and reference clones: Ps, Scots pine; Pa, Norway spruce; Pm, Douglas fir; Ld, European larch; C1-C4, reference clones for *Nitrosospira* cluster 1-cluster 4; C5 and C6, reference clones for *Nitrosomonas* cluster 5 and cluster 6; R1-R5, pure cultures for *Nitrosomonas europaea*, *Nitrosomonas eutropha*, *Nitrosomonas urea*, *Nitrosospira* sp. AHB1 and *Nitrosospira briensis*, respectively. Sequenced bands are indicated in the figure with white circles.

MPN-based estimates of AOB population densities in the four soils (Table 1) were in line with the molecular genetic analyses: AOB could be cultured from Norway spruce, Douglas fir and European larch soils, but were undetectable in Scots pine soil.

The variability in AOB population densities was extremely high, and the results must therefore be taken with caution. MPN counts from the three soils were not significantly different (Kruskal-Wallis test).

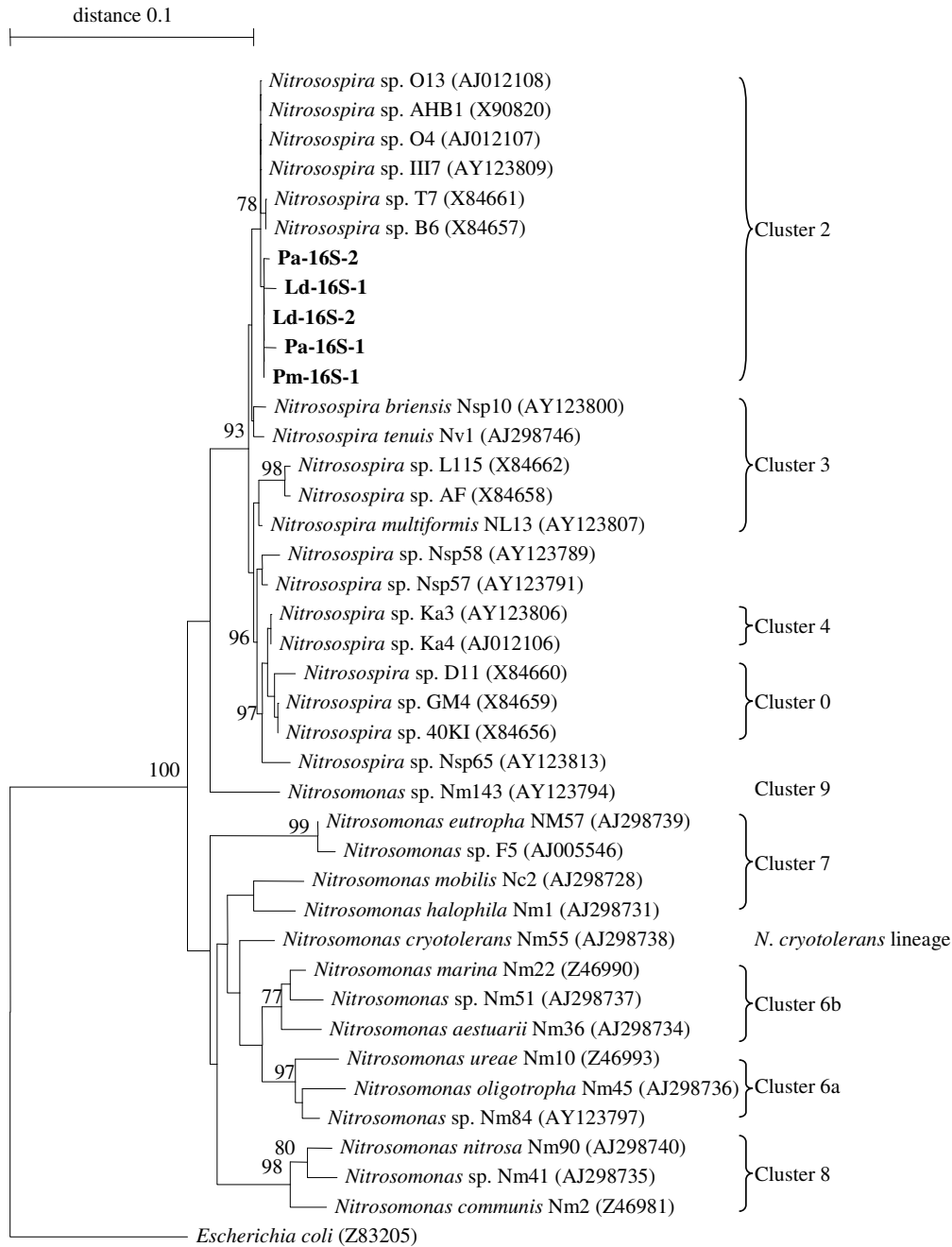


Figure 4. Neighbour-joining tree of partial 16S rRNA sequences (427 informative positions) recovered from three tree species soil samples (in bold) using CTO primers, showing the similarity between the retrieved sequences and their placement in *Nitrosospira* cluster 2. Cluster designations are those used by Koops et al. (2003). Bootstrap values are given at nodes when they exceed 75% of replicates. The GenBank accession numbers for the partial 16S rRNA sequences described in this study, designated Pa-16S-1, Pa-16S-2, Pm-16S-1, Ld-16S-1, Ld-16S-2, are DQ009867-DQ009871, respectively.

Discussion

This study showed that N transformation rates and the presence of AOB related to the variation in the C/N ratios of the soils. The F soil layer was chosen in this study, as in this layer, compared to the litter (L) layer and mineral soil, less spatial variation was observed in mineralisation and nitrification rates and temporal variation in these rates was not significant (Laverman et al., 2000).

Norway spruce, Douglas fir and European larch soils had lower C/N ratio (or higher total N) than Scots pine soil. The C/N ratio of Norway spruce soil was also found to be lower than Scots pine soil at originally similar field afforestation sites (Berg and Meentemeyer, 2002; Priha and Smolander 1997). The C/N ratio is a predictor of soil organic matter quality (Howard et al., 1998). Riha et al. (1986) stated that a high soil C/N ratio produces a strong demand for N by heterotrophic soil microbes, leaving less N available for nitrification. Our results, both the nitrification rates and the presence of AOB, are in line with this hypothesis. Several studies have observed that net nitrification rates increase with decreasing C/N ratio below a threshold value of about 22-25 (e.g. Goodale and Aber, 2001; Gundersen et al., 1998; Lovett and Rueth, 1999; Ollinger et al., 2002).

Tree species can control soil C/N ratio. Trees vary markedly in litter chemistry, and this variation can cause different rates of litter decomposition and C and N storage in soils (Hättenschwiler and Vitousek, 2000; Melillo et al., 1982; Son and Gower, 1992). Other candidates for control of soil C/N ratio include atmospheric N deposition, climate, forest history, and, in some cases, geology (Lovett et al., 2002). However, we do not expect the effects of these factors to be important in our study because all tree species studied were growing in the same forest: they were all of the same age, growing in the same soil type, at similar elevation and under comparable climatic conditions and received similar low N deposition.

Moreover, molecular genetic analysis revealed that *Nitrosospira* cluster 2 was the only sequence cluster detected in Norway spruce, Douglas fir and European larch forest soils. Thus, differences in soil properties, or tree species, did not overtly influence the composition of the AOB community. Conversely, the presence of *Nitrosospira* cluster 2 could not be detected in Scots pine soil, where net nitrification rate was low. Regarding these results, low C/N ratio (or high total N) in acidic forest soils are probably favourable for the presence of *Nitrosospira* cluster 2. This result was supported by the MPN culturing data we obtained. Although AOB are autotrophic and do not depend on organic matter input directly, differences in the magnitude or range of organic matter input may result in different or more variable rates of soil nitrogen mineralisation (Hobbie, 1996; McLaugherty et al., 1985), the main process that provides ammonium to soil AOB. Furthermore, the absence of AOB in the Scots pine soil, having a high C/N ratio, is in line with the hypothesis of Riha et al. (1986) stating that under these conditions heterotrophic bacteria out compete the AOB for ammonium. We do not believe that the absence of *Nitrosospira* cluster 2 in Scots pine soil is due to spatial heterogeneity in AOB community composition at Appelscha. Little temporal and spatial variation in AOB community composition, with the exclusive detection of a single *Nitrosospira* 16S rRNA sequence cluster, was detected in a nitrogen-saturated Scots pine forest soil (Laverman et al., 2001; 2005). *Nitrosospira* cluster 2 (as well as other

AOB) was also absent in other Scots pine forest soils receiving low N deposition, while it was present in forests in soils with high N deposition (Chapter 2).

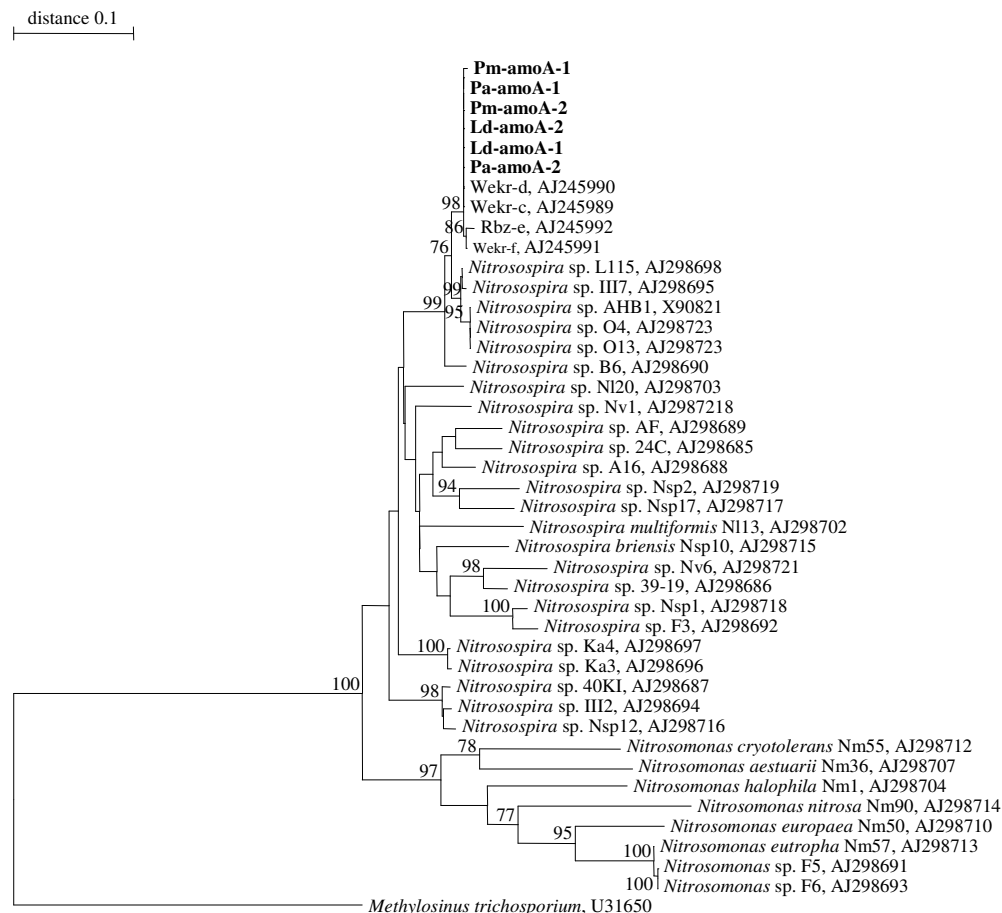


Figure 5. Neighbour-joining tree of partial *amoA* sequences (327 informative positions) recovered from three tree species soil samples (in bold) using *amoA* primers, showing the similarity between the retrieved sequences and their placement in *Nitrosospira* cluster 2 (regarding the cluster designations used by Avrahami and Conrad, 2003). Bootstrap values are given at nodes when they exceed 75% of replicates. The GenBank accession numbers for the partial *amoA* sequences described in this study, designated Pa-amoA-1, Pa-amoA-2, Pm-amoA-1, Pm-amoA-2, Ld-amoA-1, Ld-amoA-2, are DQ009876-DQ009881, respectively.

Soil acidity was not correlated to the variation in N transformation rates and the presence of *Nitrosospira* sp. cluster 2. This is in line with our previous results (Chapter 2). This could be due to the smaller range of pH found in these soils compared to other studies which found positive relationships, although presence of *Nitrosospira* cluster 2 was observed in Swedish spruce forest soils with low N deposition rate (7 kg N ha⁻¹ y⁻¹) regardless of soil pH (Bäckman et al., 2004).

In conclusion, at our study site, members of *Nitrosospira* cluster 2 dominate in acidic soils with high nitrification rates regardless of the tree species. Low C/N ratio (or high total N) of soils are probably favourable for the presence of *Nitrosospira* cluster 2. Thus, tree species can affect N transformation rates in acid coniferous forest soils with low N deposition, possibly through the species' effects on soil C/N ratios. Moreover,

this study shows that large differences in N transformation rates and presence of *Nitrosospira* cluster 2 can occur within relatively short (0.5-5 km) distances.

Acknowledgements

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CHAPTER 4

LOW NITRIFICATION RATES IN ACID SCOTS PINE FOREST SOILS ARE DUE TO pH-RELATED FACTORS

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Abstract

In a previous study, ammonia-oxidising bacteria (AOB)-like sequences were detected in the fragmentation layer of acid Scots pine (*Pinus sylvestris* L.) forest soils (pH 2.9-3.4) with high nitrification rates ($>11.0 \mu\text{g g}^{-1} \text{ dry soil wk}^{-1}$), but were not detected in soils with low nitrification rates ($<0.5 \mu\text{g g}^{-1} \text{ dry soil wk}^{-1}$). In the present study, we investigated whether this low nitrification rate has a biotic cause (complete absence of AOB) or an abiotic cause (unfavourable environmental conditions). Therefore, two soils strongly differing in net nitrification were compared: one soil with a low nitrification rate (location Schoorl) and another soil with a high nitrification rate (location Wekerom) were subjected to liming and/or ammonium amendment treatments. Nitrification was assessed by analysis of dynamics in $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ concentrations, while the presence and composition of AOB communities were assessed by PCR-DGGE and sequencing of the ammonia monooxygenase (*amoA*) gene. Liming, rather than ammonium amendment, stimulated the growth of AOB and their nitrifying activity in Schoorl soil. The retrieved *amoA* sequences from limed (without and with N amendment) Schoorl and Wekerom soils were exclusively belonging to *Nitrosospira* cluster 2. Our study suggests that low nitrification rates in acidic Scots pine forest soils are due to pH-related factors. *Nitrosospira* cluster 2 detected in these soils is presumably a urease positive cluster type of AOB.

Keywords: nitrification; liming; ammonia availability; acid Scots pine forest soils; *Nitrosospira* cluster 2

Introduction

Environmental factors, such as ammonia availability, organic matter content, and/or soil pH, influence the presence of specific types of ammonia-oxidising bacteria (AOB) and nitrification rates in coniferous forest soils (de Boer and Kowalchuk, 2001; Kowalchuk and Stephen, 2001). Specific clusters of the *Nitrosospira* group of AOB may be selected for by soil pH (reviewed in de Boer and Kowalchuk, 2001; Kowalchuk and Stephen, 2001), and it has been suggested that *Nitrosospira* cluster 2 is favoured in soils with pH values <4.2 (Stephen et al., 1996; 1998). Elevated pH, resulting from liming, enhanced the nitrification potential (Bäckman and Klemetsson, 2003), as well as growth of AOB in the fragmentation (F) layer of acid Norway spruce (*Picea abies* (L.) Karst.) forest soils (Bäckman et al., 2003). In these soils (pH 3.6–4.8), after six years of liming AOB-like sequences were detected, regardless of lime doses applied, but remained undetectable in the unlimed soils (pH 2.6). In the organic layer of a Norway spruce forest soil, liming has been found to increase the nitrification rate (Carnol et al., 2002). However, 18 months after liming the AOB communities did not differ between limed (pH 4.9) and unlimed soils (pH 3.8).

The nature of the positive effect of pH increases on nitrification was not investigated in the aforementioned studies by Carnol et al. (2002) and Bäckman and Klemetsson (2003). It is assumed that free ammonia (NH₃) rather than ammonium (NH₄⁺) is the substrate for AOB (Burton and Prosser, 2001). A higher pH shifts the equilibrium between ammonia and ammonium more towards ammonia, thus increasing ammonia availability. Ammonium itself can also be a selective factor with respect to the composition of AOB communities. Soils with high ammonium content are generally dominated by *Nitrosospira* cluster 1 or 3 and representatives of *Nitrosomonas* spp. (Kowalchuk et al., 2000a; 2000b; Nicolaisen and Ramsing, 2002). *Nitrosospira* cluster 4 is typically dominant in undisturbed and unfertilised soils with low ammonium content (Kowalchuk et al., 2000a; 2000b; Mintie et al., 2003). In the fragmentation (F) and humus (H) layers of a range of boreal forest stands in Northwestern Quebec, with original pH values of between 4.8 and 6.2, nitrification was not detected in stands with modified pH values below 4.5 (Ste-Marie and Paré, 1999). Increasing the pH of these forest soils had a positive effect on net nitrification, whereas ammonium amendment did not significantly enhance net nitrification (Ste-Marie and Paré, 1999). However, whether these changes are related to the AOB community structure in these soils is not clear as the presence of the AOB was not assessed in these studies.

The work of Ste-Marie and Paré (1999) shows that ammonium addition did not promote a significant increase in net nitrification, increase of forest floor pH had a positive effect on net nitrification while acidification depressed it. The nitrifying organisms in their soils were acid-sensitive and modification of forest floor pH had also an effect on net ammonification. This study (Ste-Marie and Paré, 1999) therefore suggests that low nitrification rates in their research location, forest soils with a modified pH above 4.5, have been attributed to pH-related factors, and not to ammonia availability. Previously, we investigated acid Scots pine forests with a pH around 3.0. Our research revealed that exclusively *Nitrosospira* cluster 2-like 16S rRNA genes were detected in the F layer of acid Scots pine (*Pinus sylvestris* L.) forest soils with high nitrification rates (11.0–31.6 µg g⁻¹ dry soil wk⁻¹) (Chapter 2). In the soils with low

nitrification rates ($<0.5 \mu\text{g g}^{-1}$ dry soil wk^{-1}), AOB-like sequences could not be detected (Chapter 2). As the original soil pH values were not significantly different between the nine investigated acidic Scots pine forest soils (pH_{KCl} 2.9-3.4), soil pH alone could not explain why AOB were not revealed in soils with low nitrification rates (Chapter 2). In contrast, a correlation was observed between C/N ratio and presence of AOB: nitrification rates were low and AOB were not detected when the C/N ratio was high (Chapter 2). This suggests that nitrogen availability might be an important factor in the occurrence of nitrification for these acidic forest soils.

The present study aimed to answer the following questions: First, do the low nitrification rates in soils relate to the absence of AOB or to unfavourable environmental factors, i.e. low soil pH and ammonia availability? Secondly, which of the environmental factors pH and ammonia availability affect nitrification the most? We tested these questions by subjecting two forest soils, strongly differing in nitrification rate, to a combination of liming and/or ammonium amendment treatments. The amendment with ammonium allowed us to determine whether a possible effect of liming on nitrification is the result of higher nitrogen availability, or is due to other pH-related factors. Net nitrification rates were determined by the increase in nitrate concentration in time and we applied a molecular fingerprinting technique using the *amoA* gene, which encodes the α subunit of ammonia monooxygenase for analyzing AOB communities. The phylogeny of the *amoA* gene has been found to correspond largely to the phylogeny of the 16S rRNA gene in AOB (Aakra et al., 2001; Kowalchuk and Stephen, 2001; Purkhold et al., 2003).

Materials and Methods

Sites description and sampling. The forest floor of Scots pine (*Pinus sylvestris* L.) stand utilized in this study was sampled in February 2005 from Schoorl (latitude $52^{\circ}43' \text{N}$; longitude $4^{\circ}40' \text{E}$) and Wekerom (latitude $52^{\circ}06' \text{N}$; longitude $5^{\circ}41' \text{E}$). Schoorl and Wekerom were chosen to represent soils with low and high net nitrification rates, respectively (Chapter 2). Further selected soil characteristics from both sites are given in Chapter 2.

At each sampling site, nine samples (15×20 cm) of the forest floor (F layer) were randomly collected from a 5×5 m plot, then randomly pooled to give three composite samples and returned to the laboratory in cooling boxes. Field-moist soils were immediately passed through a 4 mm sieve in the laboratory, homogenized by hand and then stored at 5°C , to minimise changes in initial conditions across the soils, for less than a week until further analysis.

Experimental modifications of pH and ammonium. Sub-samples of soil were brought to 68% moisture content by adding sterile demineralised water. Microcosms were setup in sterile 250 ml bottles, using aseptic techniques. Soil samples (15 g fresh weight) from Schoorl and Wekerom were put in the bottles and subjected to four different treatments, with each treatment having 15 bottles: control – no addition; liming – addition of 0.5 g CaCO_3 ; N amendment – addition of 1 ml of 25 mM $(\text{NH}_4)_2\text{SO}_4$ to enhance the NH_4^+ -N content by $99.2 \mu\text{g g}^{-1}$; liming + N amendment – addition of 0.5 g

CaCO₃ and 1 ml of 25 mM (NH₄)₂SO₄. Bottles were sealed with cotton plugs and incubated at 18 °C in the dark. Soil moisture was maintained by periodic addition of sterile demineralised water. Bottles were destructively sampled after 0, 2, 4, 10 and 12 weeks. Three bottles were sampled per treatment and per sampling occasion. Extraction and determination of NH₄⁺-N and NO₃⁻-N concentrations were carried out as described previously (Chapter 2).

DNA extraction, PCR, DGGE and cloning. Samples for AOB community analysis were taken at the start (0 weeks) and end (12 weeks) of the experiment for each treatment. DNA was extracted from approximately 0.15 g (fresh weight) sub-samples of soil using the FastDNA[®] SPIN Kit for Soil (Qbiogene, Carlsbad, CA, USA). The extracted DNA was cleaned with the Wizard DNA clean-up system (Promega, Madison, WI, USA).

The *amoA* gene fragments (approximately 500-bp) were amplified from DNA extracts in 50 µl reactions containing 400 nM primers amoA-1F-Clamp/amoA-2R-TC (Nicolaisen and Ramsing, 2002), 0.2 mM dNTPs, 10 µg BSA, 2.5 units *Taq* DNA polymerase, the buffer conditions recommended by the manufacturer, and 5 µl template. The reaction conditions were 5 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 57°C, 45 s at 72°C, and 5 min at 72°C for the last cycle. When necessary, a semi-nested PCR, comprised of an initial PCR with the amoA-1F/amoA-2R-TC primers followed by a second PCR using the amoA-1F-Clamp/amoA-2R-TC primers was carried out.

DGGE of *amoA* PCR products was performed as described previously (Nicolaisen and Ramsing, 2002), using polyacrylamide gel with a gradient of 30-70% denaturant. DNA was visualized after SYBR Gold (Molecular Probes) staining by UV transilluminating and photographed with a digital camera. Centres of bands were excised from DGGE using a sterile pipet tip and incubated overnight in 50 µl water at 4°C. PCR amplification was carried out using amoA-1F/amoA-2R-TC primers for 25 cycles as described above. PCR products of the correct size were cleaned using Wizard PCR preps. (Promega, Madison, WI, USA), ligated to the pGEM[®]-T vector and transformed in *E. coli* competent cells (Promega). Clones were screened for inserts of the correct size by PCR amplification with amoA-1F/amoA-2R-TC primers and sequenced using an ABI PRISM 3100 Genetic Analyzer.

Data analysis. Corrections were made for measured NH₄⁺-N concentrations in soils amended with NH₄⁺-N by subtracting the amount added. Data on NH₄⁺-N and NO₃⁻-N concentrations were cube root-transformed prior to statistical analysis for fulfilling the assumptions of variance analysis. A general linear model (SPSS 11.5 for Windows) was used to test the effects of sampling sites, liming, NH₄⁺-N addition and incubation times on NH₄⁺-N and NO₃⁻-N concentrations. When necessary, pairwise comparisons were made afterwards using LSD test.

Phylogenetic analysis of *amoA* sequences was conducted as described in Chapter 3. The partial sequences have been deposited in GenBank under accession numbers DQ435816-DQ435828.

Results

Changes in pH, NH_4^+ -N and NO_3^- -N concentrations. Liming caused a significant increase ($P<0.05$) in initial soil pH_{KCl} values of Schoorl and Wekerom soils from 2.8 in both soils to 3.9 and 4.0, respectively, while N amendment did not affect initial soil pH values in both soils (Table 1). pH values in soils treated with liming and liming + N amendment remained significant higher than in control soils and soils treated with N amendment throughout the 12 weeks (Fig. 1).

Table 1. Initial NH_4^+ -N, NO_3^- -N and pH_{KCl} values in Schoorl and Wekerom soils. Corrections were made for measured ammonium (NH_4^+ -N) concentrations in soils amended with ammonium by subtracting the amount added. Data represent means (standard errors of the means).

Treatment	Schoorl			Wekerom		
	NH_4^+ -N	NO_3^- -N	pH_{KCl}	NH_4^+ -N	NO_3^- -N	pH_{KCl}
	($\mu\text{g g}^{-1}$)	($\mu\text{g g}^{-1}$)		($\mu\text{g g}^{-1}$)	($\mu\text{g g}^{-1}$)	
Control	34.0 (2.81)	0.4 (0.16)	2.8 (0.03)	53.0 (1.96)	18.9 (3.21)	2.8 (0.02)
Liming	33.7 (3.05)	0.5 (0.15)	3.9 (0.05)	56.7 (1.72)	17.3 (1.55)	4.0 (0.05)
N amendment	36.6 (2.94)	0.5 (0.21)	2.8 (0.05)	67.6 (5.38)	18.3 (2.57)	2.8 (0.02)
Liming + N amendment	39.0 (4.24)	0.5 (0.16)	3.8 (0.06)	55.3 (5.46)	17.8 (2.07)	4.0 (0.06)

N amendment increased initial NH_4^+ -N concentrations in Schoorl and Wekerom soils to about 135.8 and 166.8 $\mu\text{g g}^{-1}$ dry soil, respectively, compared to the 34.0 and 53.0 $\mu\text{g g}^{-1}$ dry soil, respectively for control soils (Table 1). Liming did not affect initial NH_4^+ -N concentrations in both soils (Table 1). Initial NO_3^- -N concentration in Schoorl soil was significantly lower ($P<0.05$) than in Wekerom soil, 0.4 vs. 18.9 $\mu\text{g g}^{-1}$ dry soil (Table 1). Treatments did not affect initial NO_3^- -N concentrations in both soils (Table 1).

Statistical tests revealed significant ($P<0.05$) interaction effects between incubation times, sampling sites and liming on NH_4^+ -N production, and between incubation times, sampling sites, liming, N amendment on NO_3^- -N production. It is noteworthy that corrections were made for measured NH_4^+ -N concentrations in soils amended with NH_4^+ -N by subtracting the amount added. NH_4^+ -N production increased significantly in Schoorl control soil and soils treated with liming, N amendment and liming + N-amendment by 165.5, 141.1, 177.2 and 158.5 $\mu\text{g g}^{-1}$ dry soil, respectively, over a time period of 12 weeks (Fig. 2a). The effects of liming and liming + N amendment on NH_4^+ -N production in Schoorl soils were not significant. In contrast to NH_4^+ -N production, liming and liming + N amendment induced an increase in NO_3^- -N production in Schoorl soils (Fig 2b). NO_3^- -N production increased significantly in Schoorl soils treated with liming and liming + N amendment by 4.7 and 1.4 $\mu\text{g g}^{-1}$ dry soil, respectively, over a time period of 12 weeks (Fig. 2b). Conversely, N amendment did not affect the NO_3^- -N production in Schoorl soil, over a time period of 12 weeks the production did not increase significantly (Fig. 2b).

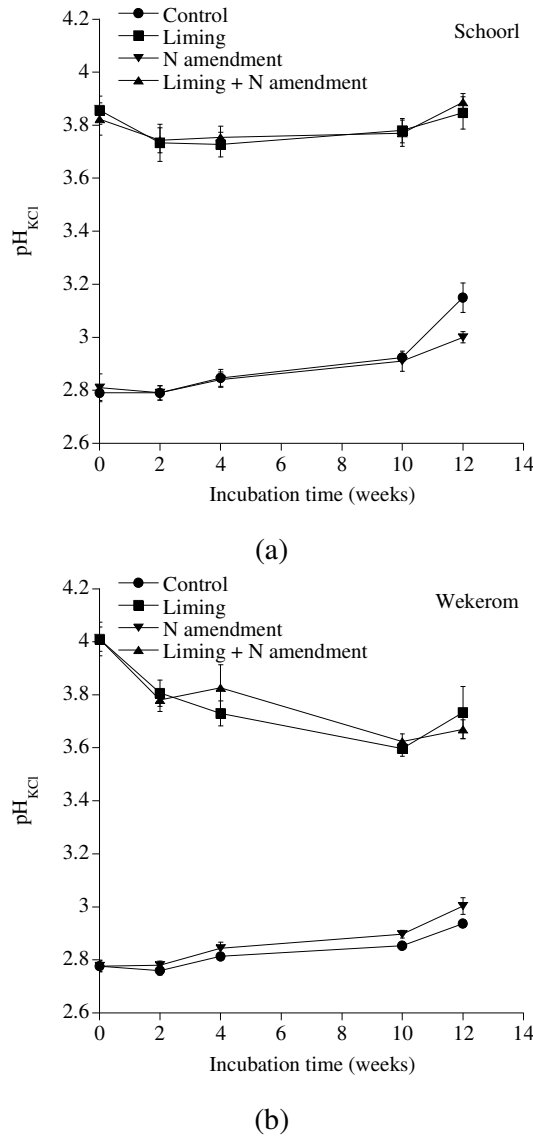
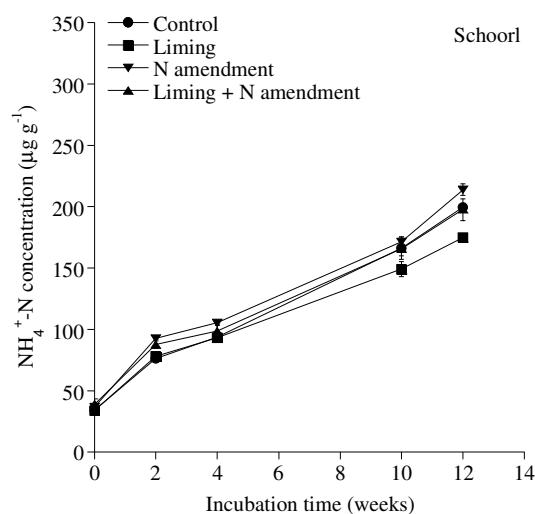
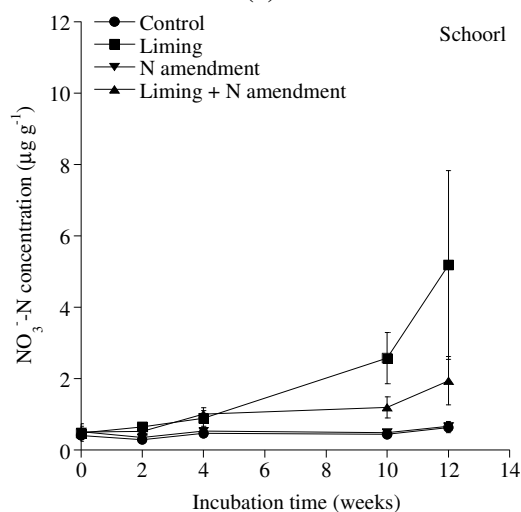


Figure 1. pH_{KCl} dynamics in Schoorl (a) and Wekerom (b) soils. Error bars represent standard errors of the means, $n = 3$. Where error bars are not shown for pH dynamics, the standard errors are smaller than the symbol.

In Wekerom soils, NH_4^+ -N production in control soil and soil treated with N amendment increased significantly by 163.3 and 215.3 $\mu\text{g g}^{-1}$ dry soil, respectively, but they decreased significantly by 33.9 and 15.2 $\mu\text{g g}^{-1}$ dry soil in soils treated with liming and liming + N amendment, respectively over a time period of 12 weeks (Fig. 3a). Furthermore, NO_3^- -N production in Wekerom soil was significantly larger than in Schoorl soil (Fig. 3b). The NO_3^- -N production increased significantly in Wekerom control soil by 48.8 $\mu\text{g g}^{-1}$ dry soil over a time period of 12 weeks (Fig. 3b). Figure 3b also shows a large increase in NO_3^- -N production as a result of liming, while N amendment did not affect NO_3^- -N production when compared to NO_3^- -N production in control soil. Liming + N amendment increased NO_3^- -N production compared to control soil, although not as much as liming alone (Fig. 3b).



(a)



(b)

Figure 2. NH_4^+ -N (a) and NO_3^- -N (b) dynamics in Schoorl soil. Error bars represent standard errors of the means, $n = 3$. Where error bars are not shown for NH_4^+ -N and NO_3^- -N dynamics, the standard errors are smaller than the symbol.

Types of AOB present. DGGE profiles only revealed a single band that co-migrated with the *Nitrosospira* sp. AHB1 reference. At the start of the experiment, this band was only detected for the Wekerom samples, while in the twelfth week of the experiment, this band was solely detected for Schoorl and Wekerom soils treated with liming and liming + N amendment (Fig. 4). Schoorl and Wekerom control soils and soils treated with N amendment for 12 weeks did not generate PCR products even though a semi-nested PCR was carried out. Wekerom control soil and soil treated with N amendment, however, still generated the expected PCR products in the tenth and fourth week of experiment, respectively. DGGE analysis revealed also for these samples only bands co-migrating with the *Nitrosospira* sp. AHB1 reference (data not shown). Analysis of duplicate samples from separate bottles, receiving the same treatment and destructively

sampled at the same time, showed that DGGE banding patterns were reproducible (data not shown).

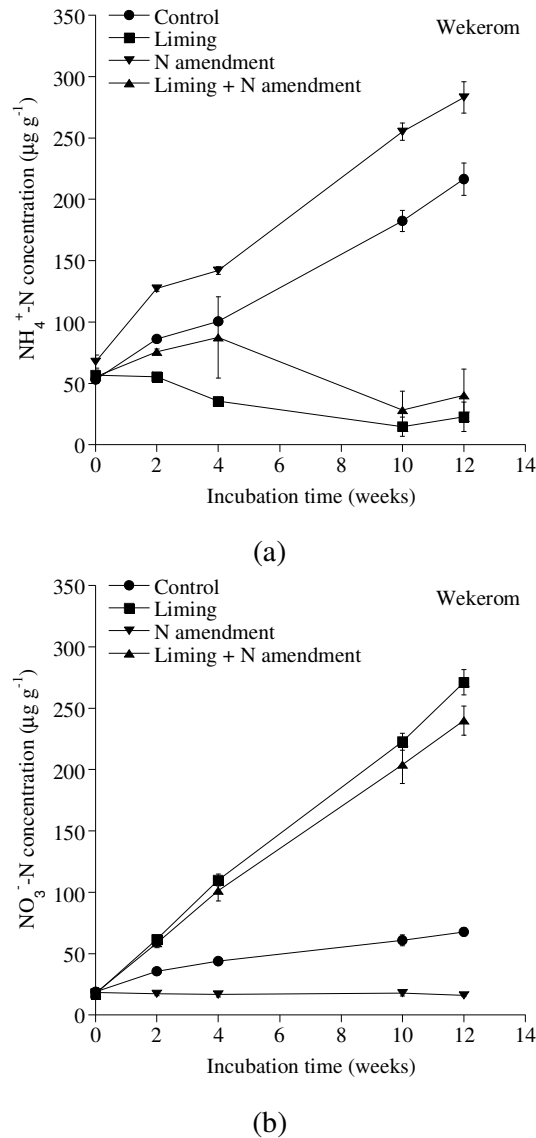


Figure 3. $\text{NH}_4^+\text{-N}$ (a) and $\text{NO}_3^-\text{-N}$ (b) dynamics in Wekerom soil. Error bars represent standard errors of the means, $n = 3$. Where error bars are not shown for $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ dynamics, the standard errors are smaller than the symbol.

Reamplification of the excised band that appeared to co-migrate with *Nitrosomonas* (indicated with a black circle in Fig. 4) for the Schoorl site treated with liming did not produce PCR products with correct size (data not shown), while reamplification of excised bands that co-migrated with *Nitrospira* sp. AHB1 gave rise to products of the correct size. We only cloned PCR products with correct size. Phylogenetic analysis of the recovered sequences derived from Wekerom soil at the start of the experiment and from Schoorl and Wekerom soils treated for 12 weeks with liming and liming + N amendment, placed all sequences in *Nitrospira* cluster 2 (Fig. 5). The similarity

between these sequences ranged from 98% to 100%. These sequences showed the closest affinity to *Nitrospira* sp. III7 and AHB1 *amoA* sequences. The similarity to these known β -subdivision AOB sequences ranged from 96% to 100%. None of the sequences clustered with *Nitrosomonas* species.

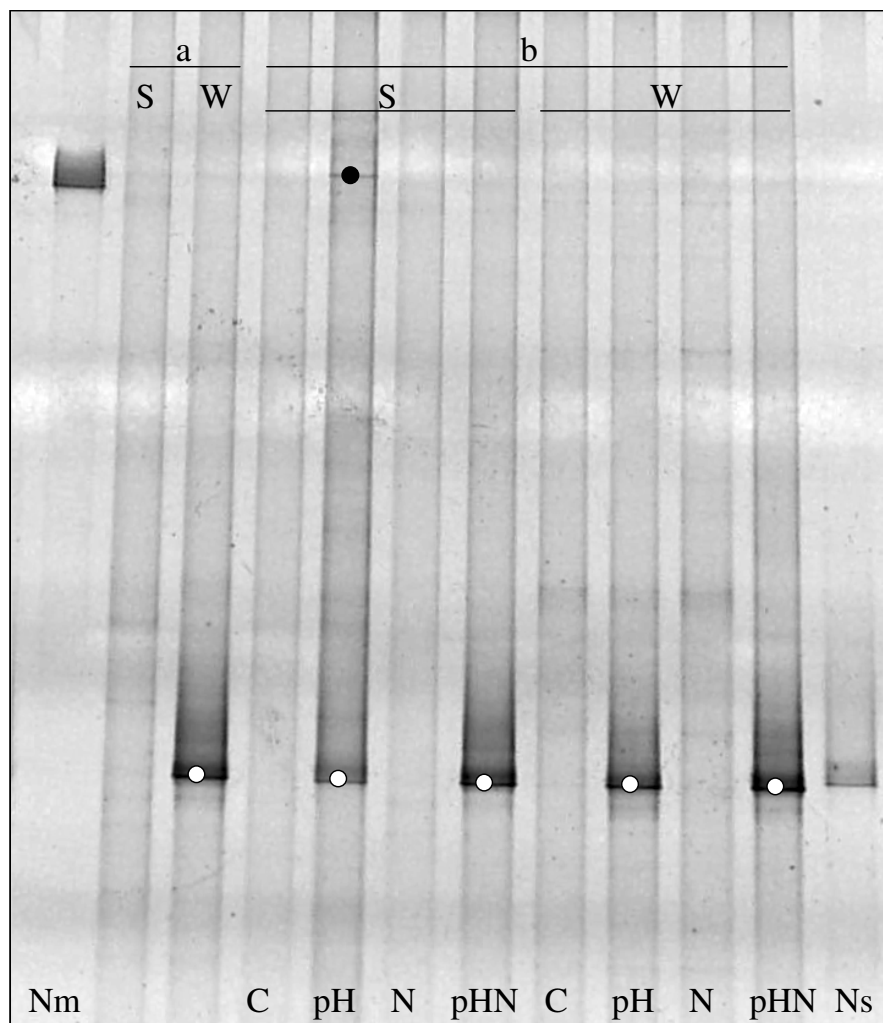


Figure 4. PCR-DGGE analysis of AOB-like *amoA* fragment from Schoorl (S) and Wekerom (W) soils at the start of the experiment (a) and in the twelfth week of experiment (b). The other abbreviations represent: Nm – *Nitrosomonas europaea*; C – Control; pH – Liming; N – N amendment; pHN – Liming + N amendment; Ns – *Nitrospira* sp. AHB1. Black circle – excised band, not given rise to correctly sized PCR product upon reamplification. Sequenced bands are indicated in the figure with white circles.

Discussion

This study demonstrated that at the start (time zero) of the experiment AOB-like *amoA* sequences, all of which were affiliated with *Nitrospira* cluster 2, were only detected in the soil with a high net nitrification rate and not in the soil with a low net nitrification rate. This is in agreement with our previous finding (Chapter 2) and consistent with previous reports of the ubiquity of *Nitrospira* in terrestrial environments (Bruns et al.,

1999; Kowalchuk and Stephen, 2001; Mintie et al., 2003; Webster et al., 2002). Regarding the presence of AOB, it should be noted that we measured the overall presence of AOB and did not attempt to quantify their numbers. Differences in numbers of AOB may explain the differences in nitrification rates. The individual steps of molecular approach (DNA extraction, PCR, and profiling) may be subject to methodological bias (von Wintzingerode et al., 1997). However, since all samples were treated similarly, these pitfalls can be considered the same for all samples, allowing between-sample comparisons.

Liming, rather than the N amendment, stimulated the growth of AOB and their associative nitrifying activity in the Schoorl soil. We conclude that the low nitrification rate in Schoorl soil is not due to the absence of AOB, but to unfavourable environmental conditions. The preferred form for uptake of ammonium is NH_3 , which can diffuse passively across the cell membrane (Burton and Prosser, 2001). As pH decreases, NH_3 ionizes to form NH_4^+ , which would require active transport across the cell membrane (Burton and Prosser, 2001). Similarly, if one adds ammonium-sulphate to soil, part of the added NH_4^+ will be converted to NH_3 as the result of thermodynamic equilibrium reactions. The comparison of the results of the experiments with Wekerom or Schoorl soil receiving N amendment to the experiments treated with liming clearly reveals that the improvement in nitrification upon liming does not relate to improved ammonia availability due to a higher pH, but to other pH-related factor(s). Both treatments increase the amount of available ammonia, but a higher nitrification rate was only observed when pH was also increased. This conclusion that the stimulation of nitrification is related to other factors than ammonia availability is further supported by the observation that a treatment with both liming and N amendment did not lead to higher nitrification rates than a treatment with liming only. In fact, nitrification rates were slightly lower when soils were both limed and receiving ammonium. The observation that forest soils with comparable pHs revealed very different nitrification rates (Chapter 2) suggests that the interplay between the occurrence of nitrification and these pH-related factors is complex. Our results do not support our hypothesis which stated that nitrogen availability, but not soil pH, determine the occurrence of nitrification for these acidic forest soils. However, our results on these low pH forest soils (pH around 3.0) correspond to previous observations on the effects of liming and N amendment on nitrification in forest soils with higher pH (pH 4.8-6.2) (Ste-Marie and Paré, 1999).

Changes in the growth of AOB in soils treated with liming and liming + N amendment were apparent from nitrification rates, since NO_3^- -N production was higher when AOB were detected. This result is in agreement with a study on the F layer of Norway spruce soil, which showed that liming induced growth of AOB (Bäckman et al., 2003). In that study, both *Nitrosospira* clusters 2 and 4 sequences were detected in the limed soil, regardless of soil pH (3.6 and 4.8), whereas AOB-like sequences were not detected in the control soil (pH 2.6) with low potential nitrification ($0.7 \mu\text{g}^{-1} \text{g}^{-1} \text{dry weight wk}^{-1}$). Using the Most Probable Number (MPN) technique, Papen et al. (2002) describe similar results; AOB were only detected four months after liming Norway spruce soil of the Höglwald Forest, Germany. Unfortunately, in that study the AOB community composition was not examined.

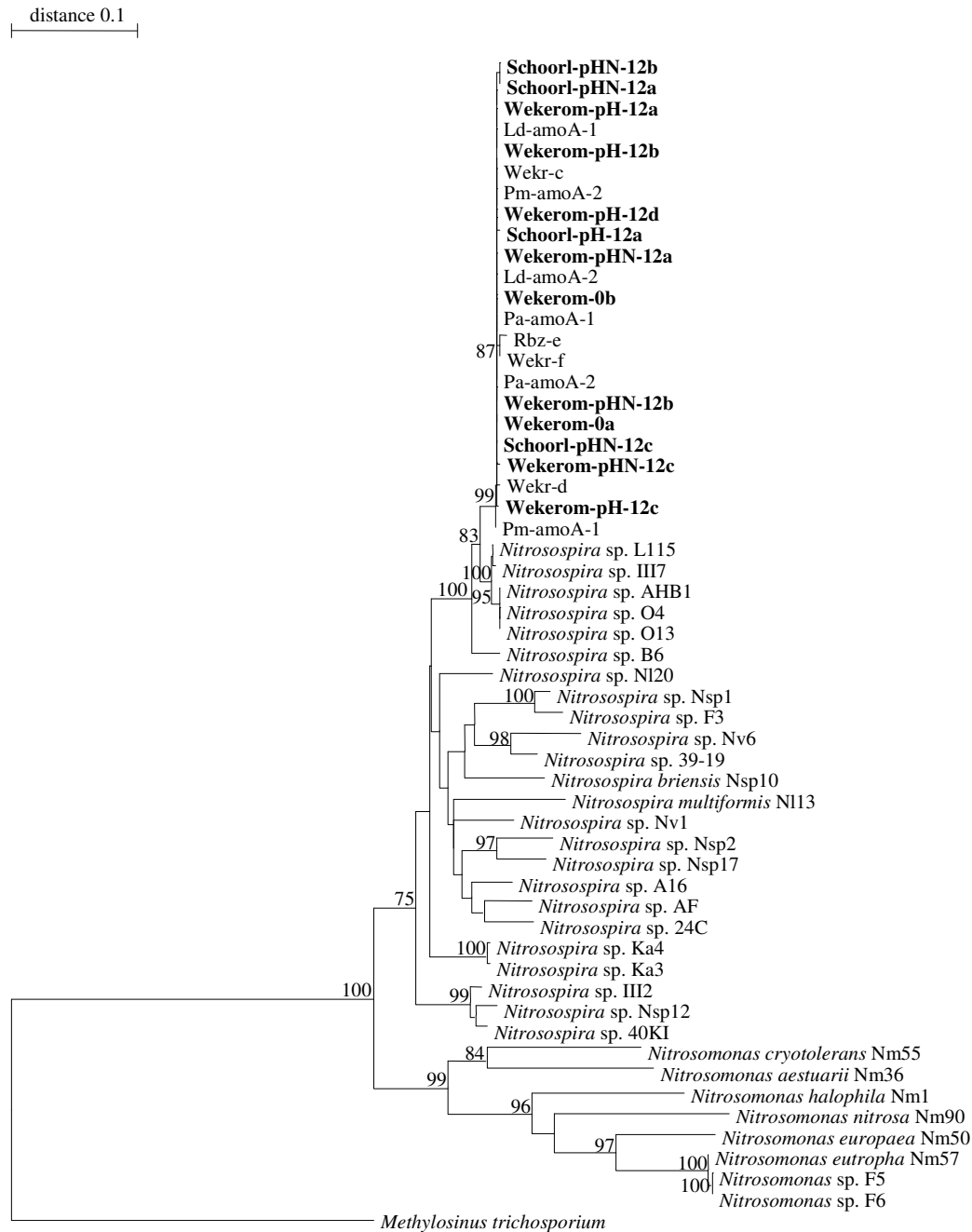


Figure 5. Neighbour-joining tree of partial *amoA* sequences (374 informative positions) recovered from untreated and treated Schoorl and Wekerom soils. Bootstrap values (based on 1000 replicates) are given at nodes when they exceed 75% of replicates. Clone sequences from this study are in bold.

Although liming enhanced growth of AOB and the nitrifying activity, our study did not reveal changes in AOB community composition upon liming. This is in agreement with earlier findings (Bäckman et al., 2003; Carnol et al., 2002; Jiang and Bakken, 1999). We detected only AOB sequences belonging to *Nitrosospira* cluster 2, despite the higher pH values (3.9 and 4.0) for both Schoorl and Wekerom soils. Moreover, the

diversity in AOB appears to be extremely low in Scots pine forest soils from Schoorl and Wekerom compared to the diversity in AOB in other coniferous forest and hardwood forest soils (Bäckman et al., 2003; Carnol et al., 2002; Compton et al., 2004; Jordan et al., 2005; Mintie et al., 2003; Yeager et al., 2005). AOB other than *Nitrosospira* cluster 2 were not detected in our study. It has been postulated that pH may select for the presence of specific groups of AOB (de Boer and Kowalchuk, 2001; Kowalchuk and Stephen, 2001). Thus, it was not surprising we only detected *Nitrosospira* cluster 2 in these two acid soils, as this AOB cluster type is known to dominate in acid soils (Kowalchuk and Stephen, 2001; Kowalchuk et al., 2000b; Laverman et al., 2001). We have previously also detected a similar low AOB diversity in acidic forest soils (Chapter 2; 3) and have discussed AOB diversity in more detail there.

Our study also revealed that AOB could not be detected in Schoorl and Wekerom soils treated with N amendment in the twelfth week of experiment. This is in contrast with previous studies which demonstrated that *amoA* genes were detected in pine (pH 3.2) and hardwood forest (pH 3.3) soils treated with N amendment, but not in control soils (Compton et al., 2004). *Nitrosomonas* may become more dominant in ammonium-rich environments or when soils are amended with fertilizer (Ceccherini et al., 1999; Koops and Pommerening-Röser, 2001; Stephen et al., 1996; Webster et al., 2002). Upon NH_4^+ -N amendment, NO_3^- -N concentrations did not increase or increased less than observed for the untreated control. We suggest that *Nitrosospira* cluster 2 detected in this study is an ammonium-sensitive AOB cluster type. It was demonstrated that nitrification by *Nitrosospira* sp. AHB1, a member of *Nitrosospira* cluster 2, did not occur at pH values below 5.5 in a pH-stat with ammonium as the sole source of energy (de Boer and Laanbroek, 1989). *Nitrosomonas* spp. sensitive to $(\text{NH}_4)_2\text{SO}_4$ have been isolated from activated sludge samples (Suwa et al., 1994). This study also revealed that NH_4^+ , rather than sulphate, was inhibitory to the growth of the isolated strains (Suwa et al., 1994). At a low external pH, with ammonium as the sole substrate, AOB are not able to maintain a sufficiently high intracellular pH. As a result, ammonia monooxygenase is not supplied with ammonia at a rate high enough to maintain viability (de Boer and Laanbroek, 1989).

It has been suggested that ureolysis provides a mechanism for nitrification in acid soils, such as the Schoorl and Wekerom soils studied here (Allison and Prosser, 1991; Burton and Prosser, 2001; de Boer et al., 1989; de Boer and Laanbroek, 1989). Urea can be taken up by AOB at a low pH (pH 4.0), through diffusion, and is then hydrolyzed intracellularly to ammonia (Burton and Prosser, 2001). *Nitrosospira* sp. AHB1 isolated from a fertilised acid heath soil could hydrolyse urea at low pH (de Boer et al., 1989). Some of the *Nitrosomonas* and *Nitrosospira* strains isolated from acid Scottish soils (Allison and Prosser, 1991) and *Nitrosospira* strains isolated from terrestrial environments, including *Nitrosospira* sp. III7 (Jiang and Bakken, 1999) were also found to be urease positive. Other studies showed that three out of four $(\text{NH}_4)_2\text{SO}_4$ -sensitive *Nitrosomonas* spp. isolated from activated sludge samples utilised urea for growth (Suwa et al., 1994) and five out of six NH_4Cl -sensitive *Nitrosomonas oligotropha* strains utilised urea for growth (Koops et al., 1991). Since all AOB-like *amoA* sequences detected in this study were affiliated with *Nitrosospira* sp. III7 and AHB1, it is suggested that *Nitrosospira* cluster 2 detected in Schoorl and Wekerom is also a

urease positive AOB cluster type. This suggestion is supported by our results that, due to the decline in availability of urea concentrations, NO_3^- -N production ceased or even stopped in control soils and soils treated with N amendment, although NH_4^+ -N was still available. This suggestion is also in accordance with the fact that AOB were detected at the start of the experiment, but could not be detected in Wekerom control and N-amended soils in the twelve weeks of experiment. This would imply that urea input, such as fertilisers and soil animals that excrete urea, may be important for nitrification in acid Scots pine forest soils.

This study therefore indicates that low nitrification rates in Scots pine forest soils are due to pH-related factors. Moreover, *Nitrospira* cluster 2 detected in these soils is presumably a urease positive AOB cluster type.

Acknowledgement

The authors thank Dr. Paul L.E. Bodelier for providing cultures of *Nitrosomonas europaea* and *Nitrospira* sp. AHB1.

CHAPTER 5

NITRIFICATION AND GENERAL BACTERIAL COMMUNITY STRUCTURE AFTER CROSS-INOCULATION EXPERIMENTS IN TWO SCOTS PINE FOREST SOILS WITH DIFFERENT NITRIFICATION RATES

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Abstract

Nitrification occurs slowly in some acid Scots pine forest soils. We examined if general microbial community structure and interactions between members of the microbial community in acid Scots pine forest soils with low nitrification rate prohibit growth of autotrophic ammonia-oxidising bacteria (AOB) and their nitrifying activity. Native and sterilised Scots pine forest soils, known to have low nitrification rates were augmented with fresh soils or soil slurries from nitrifying Scots pine forest soil, and vice versa. Augmentation with nitrifying soils induced nitrification in previously non-nitrifying soils, although no changes in bacterial community structure, as measured by 16S rRNA gene-denaturing gradient gel electrophoresis (DGGE) based community profiling, were observed. In sterilised soils, the inoculum determined the occurrence of nitrification and bacterial community structure. Our results demonstrate that low nitrification rates in acid Scots pine forest soils cannot be (solely) explained by unfavourable abiotic soil conditions. The occurrence of nitrification after augmentation of native soils known to have low nitrification rates with nitrifying soil or soil slurry also falsified our hypothesis that interactions within the non-nitrifying soil microbial community prevent the onset of the growth and activity of AOB. General bacterial community structure appears to be a too insensitive factor to relate it to nitrification rates. Overall, this research indicates that interactions between microorganisms may influence the occurrence of nitrification, but these interactions are subtle and not detectable by DGGE targeting bacteria.

Keywords: acid Scots pine forest, nitrification, microbial community structure, cross-inoculation experiments

Introduction

Ammonia-oxidising bacteria (AOB) mediate the first step in nitrification, the oxidation of ammonia to nitrite. It has been suggested that AOB are present in all environments in which nitrogen is mineralized (Koops and Pommerening-Röser, 2001; Koops et al., 2003; Kowalchuk and Stephen, 2001). Although AOB could not be detected with molecular tools in several acid (pH around 3.0) forest soils with low nitrification rates (Chapter 2; Bäckman et al., 2003; Compton et al., 2004; Tolli and King, 2005), additional research indicated that AOB were actually present but in numbers which were below the detection limits of the molecular methodologies applied (Chapter 4; Bäckman et al., 2003;).

Differences in nitrification can sometimes be explained by abiotic factors. Acid forest soils with low nitrification rates are characterised by low initial NO_3^- -N concentrations, high C/N ratios (or low total N) and low atmospheric N depositions (Chapters 2 and 4; Bäckman et al., 2003; Compton et al., 2004; Persson and Wirén, 1995; Tolli and King, 2005). Low nitrification rates in acid forest soils cannot be explained solely by soil pH, since the soil pH values were found not to differ significantly between soils exhibiting low and high nitrification rates (Chapter 2). N availability also does not constrain nitrification in these soils because large amounts of NH_4^+ -N are produced during incubation of these soils in the laboratory (Chapter 2; Booth et al., 2005; Persson and Wirén, 1995). As nitrification does not occur in some acid soils with increased NH_4^+ -N concentrations, other suppressive factors need to be considered.

Differences in nitrification may also be driven by biotic factors. Nitrification rates might be linked to the composition and structure of the soil bacterial community (Balser and Firestone, 2005; de Boer and Kester, 1996; de Boer et al., 1996; Wheatley et al., 2003). Some of its members may have specific positive or negative effects on the activity of the nitrifying bacteria, e.g. chitinolytic soil bacteria producing antibiosis against nitrifying bacteria (de Boer et al., 1996). Furthermore, AOB are generally considered poor competitors for NH_4^+ relative to heterotrophs (van Niel et al., 1993; Verhagen and Laanbroek, 1991; Verhagen et al., 1992; 1995). In competition experiments, which were conducted with mixed cultures of *Nitrosomonas europaea* and *Arthrobacter globiformis* in the presence of *Nitrobacter winogradskyi*, the nitrification process was shown to be inhibited by NH_4^+ assimilation by the more competitive heterotrophic *A. globiformis* (Verhagen and Laanbroek, 1991; Verhagen et al., 1992). Wheatley et al. (2003) have compared the structure of the eubacterial community within and between three arable fields differing in potential nitrification rates but broadly similar in the basic characteristics of the fields (soil pH, total C and total N contents) and they found that the bacterial community structure in each field was significantly different from that in the others. In contrast, molecular analyses specific to AOB suggested that the populations in all three fields were similar in types and did not vary in time (Wheatley et al., 2003).

The relationship of overall bacterial community structure to nitrification rates in acid Scots pine forest soils has not been thoroughly studied. Our aim was to examine if general microbial community structure and interactions between members of the microbial community in acid Scots pine forest soils with low nitrification rate prohibit

growth of autotrophic AOB and their associative nitrifying activity. If so, we may expect that when native soils (showing either low or high nitrification rates) are inoculated, or augmented, with a small quantity of another forest soil with contrasting nitrification activity, no significant changes in nitrification will occur. However, we would expect that when the soil microbial community is destroyed by sterilisation, the nitrifying potential of the inoculum will be able to establish itself in the sterilised soil, unless abiotic conditions in the sterilised soil prohibit this. To test these ideas, native and sterilised Schoorl soils, known to have low nitrification rates (Chapters 2 and 4), were augmented with fresh soils or soil slurries from nitrifying Wekerom soil (Chapters 2 and 4; Laverman et al., 2001), and vice versa. Nitrification rates were determined and related to changes in overall bacterial community profiles, established by 16S rRNA gene based community profiling.

Materials and Methods

Study sites and soil sampling. The forest floors of Scots pine stands at Schoorl (latitude 52°43'N; longitude 4°40'E) and Wekerom (latitude 52°06'N; longitude 5°41'E), The Netherlands, were sampled in February 2005. Previously, Nugroho et al. (Chapter 2) determined that net nitrification rates in these soils were 0.1 and 14.4 $\mu\text{g NO}_3^- \text{-N g}^{-1} \text{ dry soil wk}^{-1}$, respectively, while net ammonification rates were similar (about 22.6 $\mu\text{g NH}_4^+ \text{-N g}^{-1} \text{ dry soil wk}^{-1}$). Further details on the two forest sites used in this study are given in Chapter 2. At each sampling site, eighteen samples (15×20 cm) of the forest floor (F layer) were randomly collected from a 5×5 m plot, then randomly pooled to give six composite samples and returned to the laboratory in cooling boxes.

Table 1. Treatments applied to soils in 6-months incubations.

Soil condition	Site of origin	
	Schoorl	Wekerom
Native soil	1. Control	1. Control
	2. Inoculated with 1 g fresh Wekerom soil	2. Inoculated with 1 g fresh Schoorl soil
	3. Inoculated with 1 ml fresh Wekerom soil slurry	3. Inoculated with 1 ml fresh Schoorl soil slurry
Sterilised soil	1. Control	1. Control
	2. Inoculated with 1 g fresh Wekerom soil	2. Inoculated with 1 g fresh Schoorl soil
	3. Inoculated with 1 ml fresh Wekerom soil slurry	3. Inoculated with 1 ml fresh Schoorl soil slurry
	4. Inoculated with 1 g fresh Schoorl soil	4. Inoculated with 1 g fresh Wekerom soil
	5. Inoculated with 1 ml fresh Schoorl soil slurry	5. Inoculated with 1 ml fresh Wekerom soil slurry

Laboratory incubation. Field-moist soils were immediately passed through a 4 mm sieve in the laboratory and homogenized by hand. Three composite samples were then stored at 5°C, while other three composite samples were sterilised by 25 kGy gamma (γ) irradiation at Isotron Nederland B.V.

Sub-samples of native and sterilised soils were brought to 68% moisture content by adding sterile demineralised water. Native or sterilised soil samples (10 g fresh weight) from Schoorl and Wekerom were put in 250 ml sterile bottles, using aseptic techniques, and subjected to different inoculants (soil or soil slurry from the same or different site of

origin) as outlined in Table 1, each inoculation having 12 replicates. Soil slurry was chosen to obtain soil microorganisms at a relatively high concentration and to separate them from large soil materials that may for example contain abiotic factors affecting nitrification. Soil slurry was prepared by mixing unsterilised soil and sterile 0.1% sodium pyrophosphate (soil : solution ratio 1 : 1), shaken for 2 h on a shaker (100 rev min⁻¹) at room temperature: the mixture was allowed to settle for 15 min before the supernatant containing the desorbed bacterial cells was decanted into sterile eppendorf tubes. Bottles were sealed with cotton plugs and incubated at 18 °C in the dark. Soil moisture was maintained by periodic addition of sterile demineralised water. Destructive samplings were conducted after 0, 1, 3 and 6 months. Three bottles were sampled per treatment and per sampling occasion. Extraction and determination of NH₄⁺-N and NO₃⁻-N concentrations were carried out as described previously (Chapter 2).

DNA extraction, PCR, DGGE and cloning. Samples for analysis of microbial communities were taken at the end (6 months) of the experiment for each treatment. DNA was extracted from approximately 0.15 g (fresh weight) sub-samples of soil using the FastDNA[®] SPIN Kit for Soil (Qbiogene, Carlsbad, CA, USA). The extracted DNA was cleaned with the Wizard DNA clean-up system (Promega, Madison, WI, USA).

Bacterial 16S rRNA gene fragments were amplified from DNA extracts in 50 µl reactions containing 400 nM general eubacterial 357F-GC/518R primers (Muyzer et al., 1993), 0.2 mM dNTPs, 10 µg BSA, 2.5 units *Taq* DNA polymerase, the buffer conditions recommended by the manufacturer, and 5 µl template. The reaction conditions were 4 min at 94°C followed by 35 cycles of 30 s at 94°C, 1 min at 54°C, 1 min at 72°C, and 5 min at 72°C for the last cycle. DGGE of 16S rRNA fragments was performed using polyacrylamide gel with a gradient of 30-55% denaturant and run for 4 h at 200 V in 1× TAE buffer at a constant temperature of 60°C. DNA was visualised after SYBR Gold (Molecular Probes) staining by UV transilluminating and photographed with a digital camera. To aid statistical analysis of gels, a marker was added on the outsides and in the middle of the gels.

Data analysis. All statistical analyses were performed using the software SPSS 11.5. Because of the unbalanced design as there were 10 treatment combinations (5 levels of treatment factor inoculation and 2 levels of treatment factor site of origin) applied to sterilised soil while there were 6 treatment combinations (3 levels of treatment factor inoculation and 2 levels of treatment factor site of origin) applied to native soil, a general linear model with type IV sums of squares (Shaw and Mitchell-Olds, 1993) was used to test the effects of irradiation, site of origin and inoculation on the initial NH₄⁺-N and NO₃⁻-N concentrations and pH values. When necessary, variables were log₁₀ transformed to fulfill ANOVA assumptions.

Cumulative net mineralisation and nitrification were calculated by subtracting the initial (NH₄⁺ + NO₃⁻)-N and NO₃⁻-N concentrations at the start of the experiment from the (NH₄⁺ + NO₃⁻)-N and NO₃⁻-N concentrations in the soil during the respective incubation period (1, 3 and 6 months). Cumulative net mineralisation and nitrification were then fitted to a linear model to estimate net mineralisation and nitrification rates,

respectively. Differences between rates (regression coefficients) were tested for significance.

DGGE gel images were converted, normalized and analysed with the GelCompar 4.0 software package (Applied Maths, Kortrijk, Belgium), using the Pearson product moment correlation coefficient and the unweighted pair group clustering method with arithmetic averages (UPGMA).

Results

Initial NH_4^+ -N and NO_3^- -N concentrations and soil pH. Twenty-five kGy γ -irradiation significantly ($P<0.05$) increased initial NH_4^+ -N concentration in Schoorl soil from $58.9 \mu\text{g g}^{-1}$ to $124.4 \mu\text{g g}^{-1}$, but did not affect the initial NH_4^+ -N concentration in Wekerom soil. Initial NO_3^- -N concentration in Schoorl soil was not affected by γ -irradiation, but declined ($P<0.05$) from $43.6 \mu\text{g g}^{-1}$ to $8.1 \mu\text{g g}^{-1}$ in Wekerom soil (Table 2). Initial soil pH levels in the Schoorl and Wekerom soils were not affected by γ -irradiation (Table 2).

Table 2. Initial NH_4^+ -N and NO_3^- -N concentrations and pH values in Schoorl and Wekerom soils. Standard errors of the mean are shown between brackets, $n = 3$.

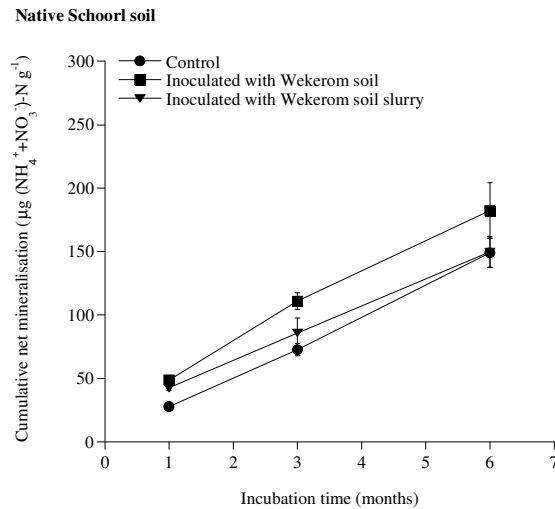
Soil condition	Inoculum	Schoorl			Wekerom		
		NH_4^+ -N ($\mu\text{g g}^{-1}$)	NO_3^- -N ($\mu\text{g g}^{-1}$)	pH _{KCl}	NH_4^+ -N ($\mu\text{g g}^{-1}$)	NO_3^- -N ($\mu\text{g g}^{-1}$)	pH _{KCl}
Native soil	Control	58.9 (0.2)	0.3 (0.0)	2.8 (0.0)	120.2 (12.0)	43.6 (6.0)	2.9 (0.0)
	Soil, allo-inoculated ^a	71.2 (0.9)	4.9 (0.7)	2.8 (0.0)	122.3 (9.7)	44.1 (4.6)	2.8 (0.0)
	Soil slurry, allo-inoculated ^a	61.3 (0.3)	3.3 (0.2)	2.8 (0.0)	115.2 (9.5)	44.6 (5.9)	2.9 (0.0)
Sterilised soil	Control	124.4 (2.4)	0.1 (0.0)	2.8 (0.0)	129.8 (1.2)	8.1 (0.5)	2.7 (0.0)
	Soil, allo-inoculated ^a	138.3 (4.5)	4.3 (0.6)	2.8 (0.1)	136.9 (1.4)	8.2 (0.5)	2.7 (0.0)
	Soil slurry, allo-inoculated ^a	123.0 (2.8)	2.8 (0.2)	2.9 (0.1)	134.0 (3.0)	8.5 (0.6)	2.7 (0.0)
	Soil, auto-inoculated ^b	127.6 (5.9)	0.2 (0.1)	2.8 (0.1)	149.2 (3.7)	11.4 (0.7)	2.7 (0.0)
	Soil slurry, auto-inoculated ^b	127.9 (5.6)	0.5 (0.0)	2.8 (0.1)	138.9 (0.5)	9.5 (0.2)	2.7 (0.0)

^a Soils were inoculated with soil or soil slurry from the other location sampled in this study, sterilised or native Schoorl soil was inoculated with Wekerom soil or soil slurry, while sterilised or native Wekerom soil was inoculated with Schoorl soil or soil slurry.

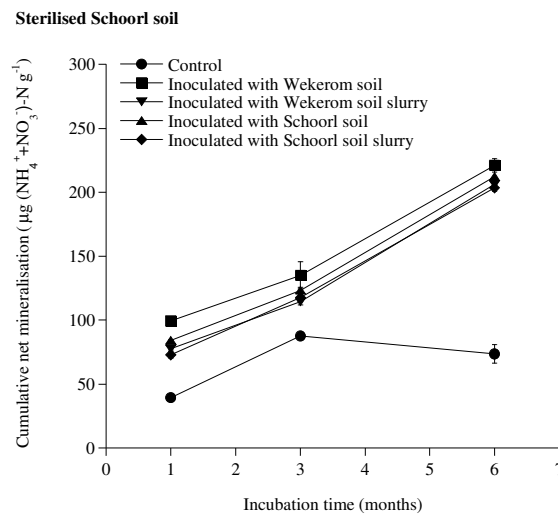
^b Soils were inoculated with soil or soil slurry from the same site of origin, sterilised Schoorl soil was inoculated with Schoorl soil or soil slurry, while sterilised Wekerom soil was inoculated with Wekerom soil or soil slurry.

Inoculations of the Schoorl and Wekerom soils with soil or soil slurry from the same site or the other site did not have a significant effect on the initial NH_4^+ -N concentrations and initial pH values (Table 2). On the other hand, initial NO_3^- -N concentrations for the Schoorl soils inoculation with Wekerom soil or soil slurry significantly ($P<0.05$) increased from 0.1 to 4.3 and $2.8 \mu\text{g g}^{-1}$, respectively (Table 2). As no such effects were observed in any of the other experiments (Table 2), the effect of Wekerom soil or soil slurry on Schoorl soils are likely attributed to the fact that the initial NO_3^- -N concentrations in native Wekerom soils are much higher than in Schoorl soils.

Effects of γ -irradiation and inoculants on net mineralisation and nitrification rates in Schoorl soil. Net mineralisation rate in native Schoorl control soil was $24.3 \mu\text{g} (\text{NH}_4^+ + \text{NO}_3^-)\text{-N g}^{-1} \text{ dry soil month}^{-1}$ (Fig. 1a). When this soil was inoculated with Wekerom soil or soil slurry, these rates were indistinguishable from the native control soil (26.5 and $21.4 \mu\text{g} (\text{NH}_4^+ + \text{NO}_3^-)\text{-N g}^{-1} \text{ dry soil month}^{-1}$, respectively (Fig. 1a). The mineralisation rate in γ -irradiated Schoorl control soil was lower than in native Schoorl control soil, at $10.2 \mu\text{g} (\text{NH}_4^+ + \text{NO}_3^-)\text{-N g}^{-1} \text{ dry soil month}^{-1}$ (Fig. 1b). When this soil was inoculated with soil or soil slurry from the same site of origin, this rate increased significantly to 25.9 and $26.3 \mu\text{g} (\text{NH}_4^+ + \text{NO}_3^-)\text{-N g}^{-1} \text{ dry soil month}^{-1}$, respectively (Fig. 1b). The rates in γ -irradiated Schoorl soil inoculated with Wekerom soil or soil slurry were also significantly higher (24.7 and $26.1 \mu\text{g} (\text{NH}_4^+ + \text{NO}_3^-)\text{-N g}^{-1} \text{ dry soil month}^{-1}$, respectively) than in the γ -irradiated Schoorl control soil (Fig. 1b) and comparable to the net mineralisation rate in native Schoorl control soil.

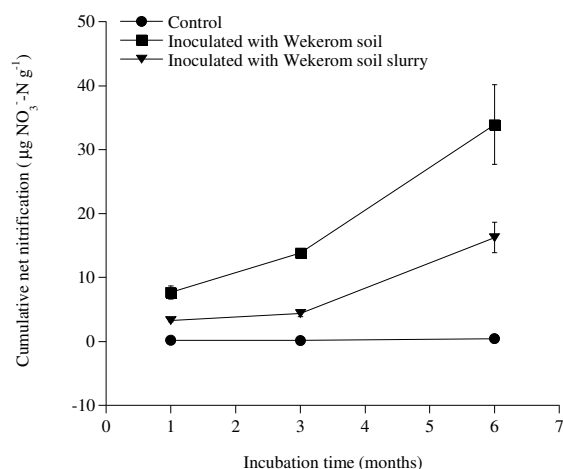


(a)



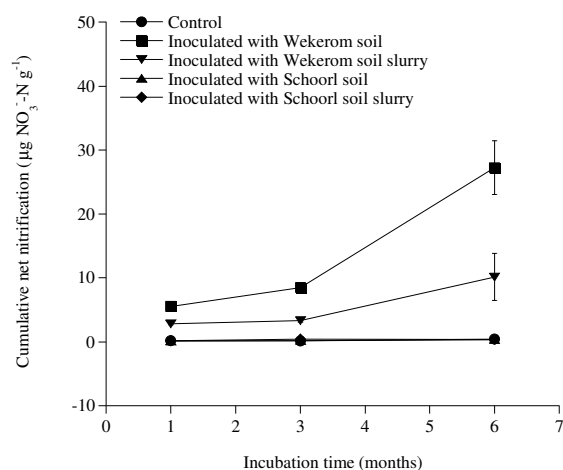
(b)

Native Schoorl soil



(c)

Sterilised Schoorl soil

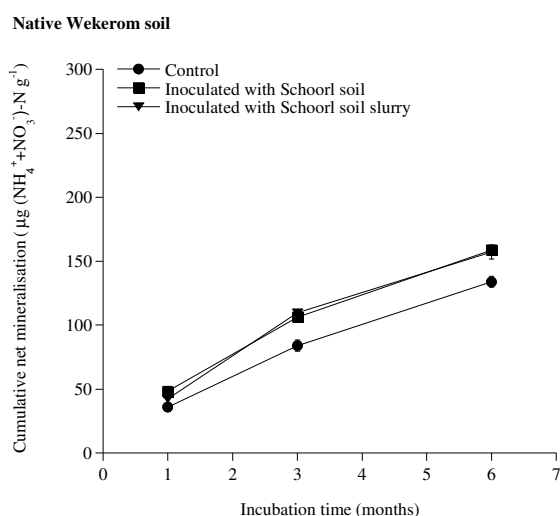


(d)

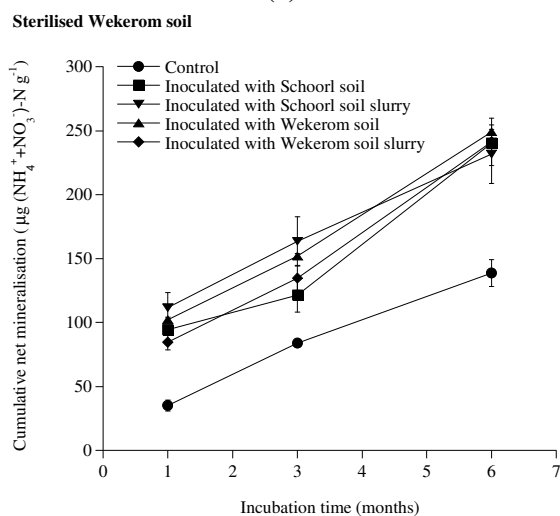
Figure 1. Cumulative net mineralisation (a and b) and nitrification (c and d) in native (a and c) and sterilised Schoorl soils (b and d), calculated by subtracting the average initial $(\text{NH}_4^+ + \text{NO}_3^-)\text{-N}$ or $\text{NO}_3^- \text{-N}$ concentrations from the $(\text{NH}_4^+ + \text{NO}_3^-)\text{-N}$ or $\text{NO}_3^- \text{-N}$ concentrations measured in the soil during the respective incubation period. Bars show standard errors of the mean, $n = 3$.

Net nitrification rate in native Schoorl control soil was relatively low, less than $0.5 \mu\text{g NO}_3^- \text{-N g}^{-1}$ dry soil month⁻¹ (Fig. 1c). Inoculating the native soil with Wekerom soil or soil slurry significantly increased nitrification rates to 5.4 and $2.7 \mu\text{g NO}_3^- \text{-N g}^{-1}$ dry soil month⁻¹, respectively (Fig. 1c). The rate in γ -irradiated Schoorl control soil was comparable to the native Schoorl soil (Fig. 1d). Inoculating this γ -irradiated soil with Schoorl soil or soil slurry did not have significant effects on this rate, while inoculating this soil with Wekerom soil or soil slurry resulted in significantly higher rates of 4.5 and $1.5 \mu\text{g NO}_3^- \text{-N g}^{-1}$ dry soil month⁻¹ respectively (Fig. 1d).

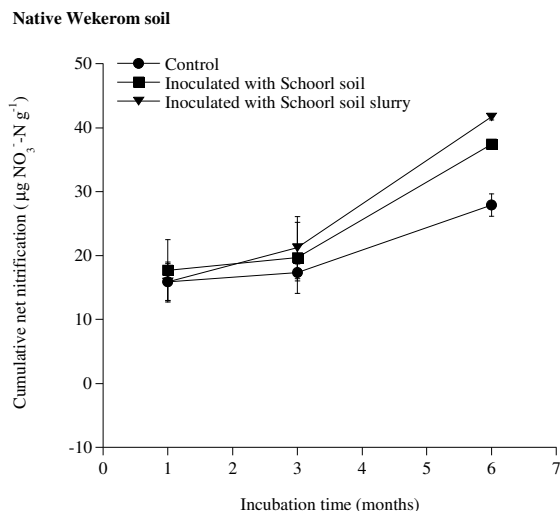
Effects of γ -irradiation and inoculants on net mineralisation and nitrification rates in Wekerom soil. Net mineralisation rate in native Wekerom control soil was $19.3 \mu\text{g} (\text{NH}_4^+ + \text{NO}_3^-)\text{-N g}^{-1} \text{ dry soil month}^{-1}$ (Fig. 2a). Inoculation with Schoorl soil or soil slurry did not affect the mineralisation rate (Fig. 2a). The rate in γ -irradiated Wekerom control soil was significantly higher at $20.5 \mu\text{g} (\text{NH}_4^+ + \text{NO}_3^-)\text{-N g}^{-1} \text{ dry soil month}^{-1}$ (Fig. 2b) than in native Wekerom control soil. When this soil was inoculated with Wekerom soil or soil slurry, the rate increased significantly to 29.6 and $31.7 \mu\text{g} (\text{NH}_4^+ + \text{NO}_3^-)\text{-N g}^{-1} \text{ dry soil month}^{-1}$, respectively (Fig. 2b). The rate in γ -irradiated Wekerom soil inoculated with Schoorl soil was also significantly ($P < 0.05$) higher at $30.0 \mu\text{g} (\text{NH}_4^+ + \text{NO}_3^-)\text{-N g}^{-1} \text{ dry soil month}^{-1}$ than in the γ -irradiated Schoorl control soil ($10.1 \mu\text{g} (\text{NH}_4^+ + \text{NO}_3^-)\text{-N g}^{-1} \text{ dry soil month}^{-1}$) (Fig. 2a). However, inoculation with Schoorl soil slurry did not increase the rate.



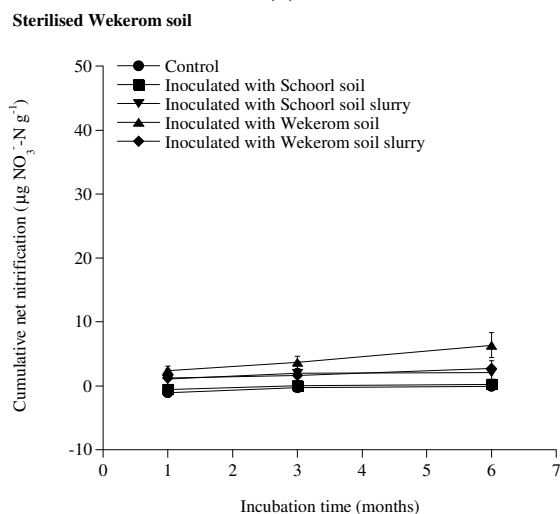
(a)



(b)



(c)



(d)

Figure 2. Cumulative net mineralisation (a and b) and nitrification (c and d) in sterilised (a and c) and native Wekerom soils (b and d), calculated by subtracting the average initial ($\text{NH}_4^+ + \text{NO}_3^-$)-N or NO_3^- -N concentrations from the ($\text{NH}_4^+ + \text{NO}_3^-$)-N or NO_3^- -N concentrations measured in the soil during the respective incubation period. Bars show standard errors of the mean, $n = 3$.

Net nitrification rate in native Wekerom control soil was $2.5 \mu\text{g NO}_3^- \text{N g}^{-1}$ dry soil month^{-1} (Fig. 2c). Inoculating this soil with Schoorl soil or soil slurry significantly increased nitrification rates to 4.1 and $5.3 \mu\text{g NO}_3^- \text{N g}^{-1}$ dry soil month^{-1} , respectively (Fig. 2c). The rate in γ -irradiated Wekerom control soil was relatively low, less than $0.5 \mu\text{g NO}_3^- \text{N g}^{-1}$ dry soil month^{-1} (Fig. 2d). Inoculating this soil with soil or soil slurry from Wekerom or Schoorl did not have significant effects on this rate (Fig. 2c).

Furthermore, the effects of γ -irradiation and inoculation of Schoorl and Wekerom soils on soil pH values during the experiment were not significant. Soil pH values increased slightly from 2.8 to 3.0 within three months in all microcosms and stayed the same till at the end of the experiment (data not shown).

Effects of γ -irradiation and inoculants on general bacterial community profiles in Schoorl and Wekerom soils. Bacterial community structures at the end of the experiment (6 months), as revealed by DGGE, differed among the two soils (Fig. 3). Cluster analysis demonstrated that sterilisation plus subsequent inoculation had pronounced impacts on the bacterial community structure in both soils. Native and sterilised Schoorl control soils were 0% similar, while native and sterilised Wekerom control soils were <40% similar (Fig. 3). The community fingerprints of sterilised Schoorl and Wekerom control soils, that did not receive an inoculum, were the most different (similarity of <25%) from all other treatments (Fig. 3). They revealed a low diversity, when judged in terms of visible DGGE bands. The bacterial fingerprints of inoculated native soils clustered were based on the origin of the soils and not based on inoculum. Bacterial communities in native Wekerom soils inoculated with Schoorl soil or soil slurry clustered with the native Wekerom control soil (similarity of 80%), while native Schoorl soils inoculated with Wekerom soil or soil slurry grouped at 74% with the native Schoorl soil (Fig. 3). The inoculated γ -irradiated Schoorl and Wekerom soils generally clustered together, separately from native Schoorl and Wekerom soils (Fig. 3). For the sterile soils, the final community composition was more related to the inoculum than to the origin of the inoculated soil: sterilised Schoorl and Wekerom soils inoculated with Wekerom soil or soil slurry clustered at 80% similarity and was different from the inoculated soils with Schoorl soil or soil slurry (Fig. 3).

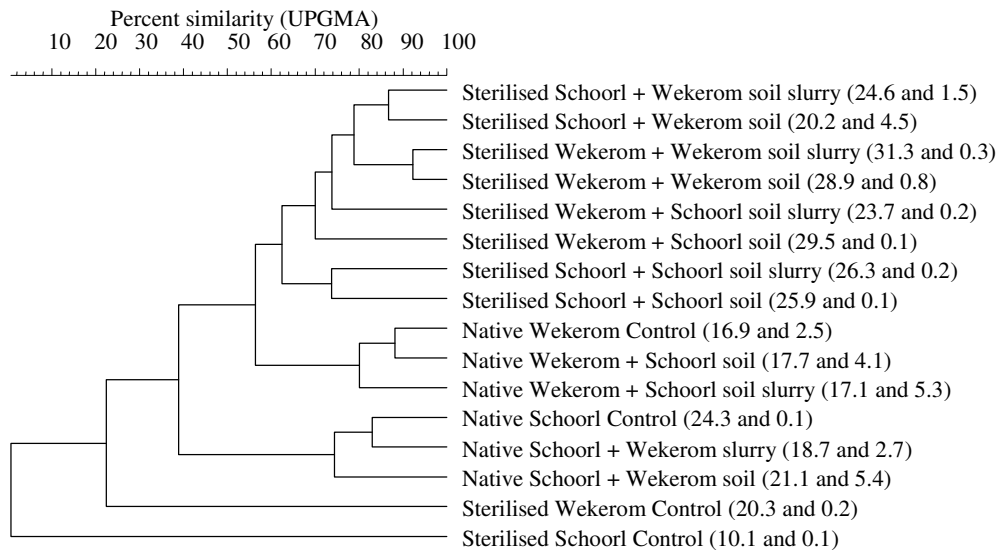


Figure 3. Dendrogram of 16S rRNA DGGE bacterial community profiles. The dendrogram was constructed using Pearson correlation and UPGMA clustering. The numbers in the brackets represent net nitrification rate ($\mu\text{g g}^{-1}$ dry soil month⁻¹).

Discussion

Our results demonstrate that low nitrification rates in acid Scots pine forest soils cannot be (solely) explained by unfavourable abiotic soil conditions. The onset of nitrification was observed after native or sterilised Schoorl soils, which in its native state hardly

showed nitrification, were inoculated with fresh soil or soil slurry from the nitrifying Wekerom location. The rates of nitrification in the augmented Schoorl soils were comparable to or higher than those in native Wekerom soils. This indicates an enhanced growth and activity of ammonia-oxidising bacteria (AOB) upon inoculation, since 10% (w/w) of fresh Wekerom soil or soil slurry was inoculated into Schoorl soils and the nitrification rates were much higher than just 10% of the rate found in Wekerom soil. In fact in many of the Schoorl soils inoculated with Wekerom soil, the nitrification rates were higher than in the Wekerom control soil. Support for a limited role of abiotic factors in the occurrence of nitrification and with respect to overall bacterial community structure also comes from the experiments on the sterilised soils. Here, the occurrence of nitrification and community structure were determined mainly by the inoculum and not by the origin of the soil that was inoculated.

The occurrence of nitrification in native Schoorl soils augmented with Wekerom soil or soil slurries also falsified our hypothesis that interactions within the non-nitrifying Schoorl microbial community prevent the onset of the growth and activity of AOB. However, despite the occurrence of nitrification the bacterial community structure did not change to become Wekerom-like, suggesting that minor changes in community structure were sufficient to result in nitrification. Thus, nitrification can be induced in non-nitrifying soils by augmentation with soils or soil slurries from nitrifying forests. In our experiments, the reverse was not observed: when Wekerom soil was inoculated with Schoorl soils or soil slurries, nitrification was not inhibited. This indicates that abiotic factors or microorganisms that can inhibit nitrification could not establish themselves in the Wekerom soils or were not even present in the non-nitrifying Schoorl soil in the first place. In fact, we found higher nitrification rates when native Wekerom soil was inoculated with Schoorl soil or soil slurry ($>4.0 \mu\text{g g}^{-1}$ dry soil month⁻¹) than in the native Wekerom control ($2.5 \mu\text{g g}^{-1}$ dry soil month⁻¹), while the bacterial community structure remained the same as the native Wekerom control.

Also the experiments on the sterilised soils indicated that general community structure is a too insensitive factor to analyse subtle interactions related to nitrification. The community profiles of these soils clustered inoculum-specific and the inoculum also determined whether nitrification occurred or not. However, the sterilised soils inoculated with Wekerom soil or soil slurry revealed large differences in nitrification rates while their bacterial community structures were quite similar. Laverman et al. (2005) were also unable to observe a clear relationship between bacterial community structure and nitrification rates when they compared a large number of soil samples from a single forest. The present study also showed that the general bacterial community structure after 6 months of incubation was not yet comparable to the general bacterial community structure in the original soils.

Overall our data, as well as those of others (Balser and Firestone, 2005; Cookson et al., 2005; Laverman et al., 2005) suggest that only the activity of a minor part of the microbial community can affect nitrification. General community profiling techniques such as DGGE, and subsequent clustering of these profiles, appear to be too insensitive to detect these minor bacterial populations. More sensitive methods are required to detect these changes, such as 16S rRNA-based taxonomic microarrays (Sanguin et al., 2006) or suppressive subtractive hybridization-based approaches (Galbraith et al., 2004)

to reveal genes specific for the Schoorl soils which are nitrifying after adding Wekerom soil.

Acknowledgement

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CHAPTER 6

GENERAL DISCUSSION

When the experimental work described in this thesis started, nitrification was assumed to be rate-controlled by the activity of ammonia-oxidising bacteria (AOB). Therefore, AOB were considered to play a crucial role in the nitrogen cycle and nitrification in particular, and AOB were the main subject of the microbiological analysis described in this thesis. The general aim of this thesis was to elucidate the relation between the presence of AOB, environmental factors, and nitrification rates in the fragmentation layer of acid (pH < 3.0) coniferous forest soils. Especially, the question why some acid forests showed nitrification while others did not was addressed in this thesis.

Nitrification tests using soil incubations showed that nitrification occurred readily in some soils, but slowly in others. This allowed drawing relationships between nitrification rates and various aspects of microbial community composition and abiotic environmental conditions (Table 1). Interactions among these factors resulted in either antagonistic or synergetic effects, thus exerting a different effect than each factor alone as will be discussed below in detail.

Table 1. Summary of the effects of biotic and abiotic factors on the occurrence of nitrification in acid coniferous forest soils, with reference to the chapters in this thesis where these effects were observed. The column 'Effect on nitrification' indicates whether particular factor can influence nitrification rates.

Environmental factor	Effect on nitrification	Chapter
Presence of AOB	No	2, 3, 4
Microbial interactions	Yes	5
C/N ratio	Yes	2, 3, 4
Atmospheric N deposition	Yes	2, 3
Tree species	Yes	3
Geographical location	No	2, 3
Soil pH	Yes	2, 4
Ammonia availability	No	2, 3, 4

Nitrosospira cluster 2 was detected as the sole AOB in five forest soils that showed high nitrification rates ($>11 \mu\text{g g}^{-1}$ dry soil wk^{-1}) (Chapter 2). Conversely, AOB communities could not be detected in four forest soils with low nitrification rates ($<0.5 \mu\text{g g}^{-1}$ dry soil wk^{-1}). Similar observations were made in the subsequent Chapters 3 and 4; soils with high nitrification rates revealed solely the presence of *Nitrosospira* cluster 2, while in soils with low nitrification rates AOB were not detectable. It has been suggested that low pH selects for *Nitrosospira* cluster 2 (Kowalchuk and Stephen, 2001). This cluster type has been found in coniferous forest soils in Europe (Chapters 2-3; Bäckman et al., 2003; 2004; Carnol et al., 2002, Hastings et al., 2000; Laverman et al., 2001; 2005;) and North-America (Compton et al., 2004; Jordan et al., 2005; Yeager et al., 2005), while there are also indications that they are present in Asia (Krave, 2002).

The presence of *Nitrosospira* cluster 2 and differences in nitrification rates between these nine Scots pine forest soils examined in Chapter 2, and the Appelscha forests containing four different tree species in Chapter 3, correlated with soil C/N ratio. *Nitrosospira* cluster 2 was exclusively detected in forest soils with C/N ratios below 26

(Chapters 2-3). The propensity for forest soils to exhibit nitrification has been linked to the C/N ratio by many studies (Bengtsson et al., 2003; Goodale and Aber, 2001; Lovett et al., 2004; Persson et al., 2000; Rueth et al., 2003; Ross et al., 2004). Low to negligible nitrification rates have been found above a C/N ratio between 22 and 25. The results described in Chapter 2 and 3 are in line with these publications and indicate that the lack of nitrification is correlated with the inability to detect AOB in these soils.

Tree species, through their effect on soil C/N ratios, were also found to affect the presence of *Nitrosospira* cluster 2 and their nitrifying activity (Chapter 3). Whenever AOB were present, the AOB community composition appeared to be similar and rather simple. *Nitrosospira* cluster 2 was detected in spruce, fir and larch forest soils. However, this cluster could not be detected in pine forest soils, where nitrification rate was low. The four tree-specific soils studied in Chapter 3 were all sampled from forests near Appelscha and located within a few kilometers from each other. Therefore, differences in nitrification rates between the nine Scots pine forest soils studied in Chapter 2 were not related to geographic distances between these forests which were located throughout the Netherlands as well as Finland.

Atmospheric nitrogen deposition rather than geographical location appeared to be a more important affecting nitrification and the presence of AOB in the nine Scots pine forest soils studied in Chapter 2. AOB could not be detected and nitrification rates were nearly zero in soils receiving relatively low, according to Dutch standards (around 21 kg N ha⁻¹ y⁻¹) atmospheric nitrogen deposition. AOB-like sequences could neither be detected in acid coniferous forest soil profiles with low nitrogen deposition (14.8 kg N ha⁻¹ y⁻¹) at a site in southwestern Sweden (Bäckman et al., 2003). The nitrification rate in this soil was nearly zero (Bäckman et al., 2003). Another study showed that nitrification rates were consistently higher at a high deposition (59.8 kg N ha⁻¹ y⁻¹) site than at low deposition (19.0 kg N ha⁻¹ y⁻¹) site in southern California (Fenn et al., 2005). These observations are in line with the results described in this thesis. However, the study described in Chapter 3 revealed that low atmospheric nitrogen deposition was not likely to be the sole factor determining nitrification. The four different soil types studied there came from around Appelscha, a location with low atmospheric nitrogen deposition. Still, three out of four forest soils revealed nitrification and the presence of AOB.

This thesis (Chapter 4) revealed that low nitrification rates observed for some soils were not caused by the complete absence of AOB nor solely by C/N ratios but also by additional abiotic factors (unfavourable environmental conditions). Liming (to increase pH) and liming plus nitrogen amendment increased nitrification in soils that were known to have low nitrification rates. Conversely, nitrogen amendment did not affect nitrification rates in these soils. These results suggested that pH related factors, other than the effect of pH on ammonia availability (described below), had negative effects on the growth of AOB and their associative nitrifying activity in non-nitrifying soils. This thesis (Chapter 4) also indicated that C/N ratio was not the sole factor affecting nitrification rates. In soils treated with liming only, the effect of liming on C/N ratio was not observed, but still nitrification increased. On the other hand, when only ammonium was added, C/N ratio dropped but this did not induce nitrification. Changes in the growth of AOB in soils treated with liming and liming plus nitrogen amendment were apparent from the observed nitrification rates, since nitrification rates were higher when

AOB were detected. A previous report also showed that liming induced growth of AOB in the fragmentation layer of Norway spruce soil (Bäckman et al., 2003). In fact, liming and nitrogen amendment increased the amount of available ammonia, the substrate for ammonia monooxygenase, as the result of thermodynamic equilibrium reactions (Suzuki et al., 1974). However, a higher nitrification rate was only observed when pH increased, showing that ammonia availability was not the factor limiting nitrification. This result is in agreement with previous observations on the effects of liming and nitrogen amendment on nitrification in forest soils with higher pH (pH 4.8-6.2) (Ste-Marie and Paré, 1999). This is also in line with the results described in Chapters 2 and 3. Ammonia availability ($5.1\text{--}106.1\ \mu\text{g NH}_4^+\text{-N g}^{-1}$ dry soil) in forest soils with comparable pH (pH 2.9-3.4) was not correlated to the presence of *Nitrosospira* cluster 2 and their nitrifying activity.

The occurrence of nitrification after augmentation of native soils that were known to have low nitrification rates with nitrifying soil or soil slurry falsified the hypothesis that interactions within the non-nitrifying soil microbial community prevent the onset of the growth and activity of AOB (Chapter 5). This chapter also indicated that interactions between microorganisms may influence the occurrence of nitrification, but these interactions were subtle and not detectable by denaturing gradient gel electrophoresis (DGGE) targeting bacteria. Overall, this thesis as well as the research of others (Balser and Firestone, 2005; Cookson et al., 2005; Laverman et al., 2005) suggests that only the activity of a minor part of the microbial community can affect nitrification. For example, Balser and Firestone (2005) and Cookson et al. (2005) found that the phospholipid fatty acid (PLFA) marker for cyclopropyl explained the variance in the relationship between PLFA guilds and nitrification rate. Laverman et al. (2005) found that nitrification in a pine forest was likely related to ammonifying microorganisms.

Are other ammonia-oxidising microorganisms contributing to the differences in nitrification rates in acid coniferous forest soils?

Till very recently, ammonia oxidation was considered to be performed largely by autotrophic ammonia-oxidising bacteria (AOB) (Purkhold et al., 2000). This thesis showed that AOB are also present in acid coniferous forest soils with low nitrification rates but are difficult to detect (Chapter 4). AOB could be easily detected in all soils with high nitrification rates (Chapters 2-4). This study and a large number of other studies, applying specific inhibitors of autotrophic nitrification (Burns and Murdoch, 2005; De Boer et al., 1992; Laverman et al., 2000; Martikainen et al., 1993; Paavolainen and Smolander, 1998; Pedersen et al., 1999; Pennington and Ellis, 1993; Rudebeck and Persson, 1998; Ross et al., 2004; Stams et al., 1990; Stark and Hart, 1997), have revealed that heterotrophic nitrification does not play an important role in acidic coniferous forest soils.

However, Jordan et al. (2005) speculated that undefined heterotrophic nitrifiers contributed to high nitrification rates in nitrogen-impacted forest soils. They showed that although the composition and abundance of autotrophic AOB populations were not significantly different across the nitrogen-saturated gradient, inhibition experiments indicated that AOB were not the major contributors to nitrification process in these

forest soils. Their forest soils were characterised by high atmospheric nitrogen deposition ($82\text{--}148.9 \text{ kg N ha}^{-1} \text{ y}^{-1}$), low pH (pH of 3.4–4.7) and high ammonia availability ($1.1\text{--}3.0 \text{ mg NH}_4^+\text{-N g}^{-1} \text{ dry soil}$) (Jordan et al., 2005). The question that arises from these discoveries is whether other autotrophic ammonia-oxidising microorganisms contribute (more than AOB) to the differences in nitrification rates in acid soils, including acid coniferous forest soils.

Recently, a unique ammonia oxygenase gene was discovered in an archaeal-associated scaffold from the Sargasso Sea (Venter et al., 2004). Highly similar *amoA* and *amoB* sequences and a linked *amoC* gene were identified in this metagenomic study, suggesting that some Archaea may be capable of performing chemoautotrophic nitrification (Venter et al., 2004). The possibility that some Archaea can carry an *amoA* was confirmed by Schleper et al. (2005), who found a Sargasso Sea-like *amoA* homolog on a 43-kb genomic fragment that also harboured a 16S rRNA gene from soil group 1.1b Crenarchaeota. This finding suggested that Archaea capable of ammonia oxidation may be also present in soils (Schleper et al., 2005). A definite link between this novel *amoA* and archaeal ammonia oxidation was only recently established by cultivation of an ammonia-oxidising member of the marine group 1.1a Crenarchaeota, the first cultivated representative from this dominant archaeal lineage (Könneke et al., 2005). Recent research has revealed that ammonia-oxidising Archaea (AOA) are widespread, abundant and active in grassland soil (Treusch et al., 2005) and in pristine and agricultural soils with pH of 5.5–7.3 (Leininger et al., 2006). They may possibly be more important in ammonia oxidation than AOB (Leininger et al., 2006).

Recent studies confirmed that Crenarchaeota are autotrophic and derive their carbon from CO_2 (Hallam et al., 2006; Ingalls et al., 2006). However, the potential of Crenarchaeota to function either as a strict autotroph or as a mixotroph utilizing both CO_2 and organic material as carbon sources has also been suggested (Hallam et al., 2006; Ingalls et al., 2006). The role of Crenarchaeota in global nitrogen cycling has been suggested recently (Venter et al., 2004; Francis et al., 2005; Schleper et al., 2005; Treusch et al., 2005). Although Crenarchaeota are considerably more abundant than characterised AOB populations, it remains to be shown, however, if all Crenarchaeota detected in soils derive their energy primarily by ammonia oxidation. If so, this raises the question in how far AOA are present and important for nitrification in acidic forest soils. Moreover, how does their contribution to nitrification compare to nitrification by AOB?

Urea hydrolysis at low pH by ammonia-oxidising Archaea (AOA)

There is strong evidence that autotrophic nitrification in low pH environments are carried out by ammonia-oxidising bacteria (AOB) capable of using urea as a source of ammonia (Burton and Prosser, 2001; Koops and Pommerening-Röser, 2001; Koops et al., 1991). Currently, AOA have been found to be predominant among ammonia-oxidising prokaryotes in twelve pristine and agricultural soils, with pH ranging from 5.5 to 7.3 (Leininger et al., 2006) thus in soils with higher pHs than the ones studied here. The *amoA* gene copies of Archaea were up to 3,000-fold more abundant than bacterial *amoA* genes. This recent finding raises the question whether AOA also contribute to the

nitrogen cycle in more acidic ($\text{pH} < 3.0$) coniferous forest soils. If so, are AOA, like AOB, capable of using urea, present in acidic soils, as a source of energy?

Since urea is a rather small and uncharged molecule, it is generally assumed that urea passes the cell-membrane by diffusion (Jiang and Bakken, 1999; Mobley and Hausinger, 1989). However, it is also suggested that urea is actively translocated in AOB cells when urea concentration is low (e.g. in the micromolar range) (de Boer and Laanbroek, 1989; Pommerening-Röser and Koops, 2005). This is then hydrolysed intracellularly to ammonia.

Recently, the potential of AOA to oxidise various reduced nitrogen compounds including ammonia and urea has been indicated (Hallam et al., 2006). Like AOB, AOA have an active uptake system for urea as shown by the genome of the marine crenarchaeote *Crenarchaeum symbiosum* which has several genes encoding homologues of urease and urea transporters (Hallam et al., 2006). Several AOB, including *Nitrosospira* sp. NpAV and *Nitrosococcus oceani* encode functional urease operons with related subunit composition to the urease observed in the *Crenarchaeum symbiosum* fosmid sequences (Koper et al., 2004). In *Nitrosospira*, urease provides a complementary source of ammonia and CO_2 for chemolithoautotrophic growth in low pH environments and a mechanism for neutralizing the surrounding acidic microenvironment (Burton and Prosser, 2001; Koper et al., 2004). Similarities between AOA and AOB in the abilities to actively take up urea and hydrolyse lead to the hypothesis that AOA may also contribute to the nitrification process in acidic ($\text{pH} < 3.0$) coniferous forest soils using reduced nitrogen compounds, including urea and ammonia, as cellular energy sources. However, even if AOA were to be detected in acid soils, their presence cannot offer an explanation on why some forest soils nitrify, while others do not: in all forest soils that revealed high nitrification rates AOB were detected, while in forest soils with low nitrification AOB were not detectable. It might therefore be that the same factors that affect AOB and their nitrifying activity also affect AOA. To test this suggestion but also to determine and understand the relative contribution of AOB and AOA to nitrification in acidic forest soils, biochemistry, physiology and ecology of AOA should be examined and compared to that of AOB.

Urea may play an important role in nitrification in acidic soils, as it has been shown that AOB and AOA are capable of utilising urea as an energy source and urea-utilisation neutralizes acidic (micro-)environments. In the microcosm experiments described in Chapter 4 nitrification tended to stop after several weeks of incubation, which possible related to the lack of urea-producing soil fauna in these experiments. This provides an additional direction for future research, i.e. the control of urea-producing soil animals on nitrification. Further research using isotopically labeled urea and urea-producing organisms in nitrification as compared to ammonia and ammonifying organisms is needed.

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